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Stabilni farmacevtski pripravek, ki vsebuje eritropoietin

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ZAHTEVA ZA PODELITEV PATENTA

1. Naslov za obveščanje:

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Potrdilo o prejemu prijave
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Žig urada in podpis:

2. Prijavitelj (priimek, ime in naslov, za pravne osebe firma in sede):
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Stabilni farmacevtski pripravek, ki vsebuje eritropoietin

6. Podatki o zahtevani prednostni pravici in podlagi zanjo:

7. Dodatne zahteve:
- ☐ prijava je za patent s skrajšanim trajanjem
- ☐ predhodna objava patenta po preteku ____ mesecev
- ☐ prijava je izločena iz prijave številka: _____

8. Izjava:
- ☐ izjava o skupnem predstavniku:

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9. Priloge:

- [x] opis izuma, ki ima __16__ strani
- [x] patentni zahtevek (zahtevki), ki ima(jo) __2__ strani; število zahtevkov: _15___
- [x] skice (če so zaradi opisa izuma potrebne); število listov: __7__
- [x] povzetek
- [ ] potrdilo o plačilu prijavne pristojbine
- [ ] potrdilo o deponiranju biološkega materiala, če gre za izum, ki ga ni mogoče drugače opisati
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- [ ] generalno pooblastilo zastopniku je deponirano pri uradu pod št.: _____
- [ ] potrdilo o razstavni prednostni pravici
- [ ] podatki o drugih prijaviteljih
- [x] podatki o drugih izumiteljih
- [ ] prikaz zaporedja nukleotidov ali aminokislin v opisu
- [ ] prijava je bila predhodno posredovana po faksu ali v elektronski obliki
- [ ] _____

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Naziv izuma

Stabilni farmacevtski pripravek, ki vsebuje eritropoietin

Področje tehnike

Predloženi izum se nanaša na nov stabilni tekoči farmacevtski pripravek, ki vsebuje eritropoietin (EPO).

EPO je glikoproteinski hormon, ki regulira tvorbo eritrocitov v sesalskem organizmu. Deluje kot rastni in/ali diferenciacijski faktor na zarodne celice kostnega mozga in povzroči njihovo diferenciacijo in proliferacijo v eritrocite.

Bistvo predloženega izuma je nov stabilni tekoči farmacevtski pripravek, ki vsebuje EPO, stabilizira EPO in ne vsebuje dodatkov humanega ali živalskega izvora (npr. serumskih proteinov). Farmacevtski pripravek je pripravljen v farmacevtsko sprejemljivem pufrskem sistemu in vsebuje povidon (PVP) kot stabilizator. Opcijsko farmacevtski pripravek dodatno vsebuje eno ali več farmacevtsko sprejemljivih pomožnih snovi. Farmacevstki pripravek, ki je predmet izuma, je farmacevtsko sprejemljiv za parenteralno aplikacijo (npr. za intramuskularno, subkutano in/ali intravenozno aplikacijo) in je primeren za uporabo v medicini.

Stanje tehnike

EPO deluje kot rastni in/ali diferenciacijski faktor na zarodne celice kostnega mozga - povzroči njihovo diferenciacijo v eritroblaste, iz katerih se razvijejo eritrociti (Goldwasser in sod., *J. Biol. Chem.,* 249,4202-4211,1974). Njegova sinteza poteka pri odraslih v ledvicah (Sherwood in sod., *Endocrinology*, 103, 866-870, 1978) pri zarodkih pa v jetrih (Zanjani in sod., *J. Lab. Clin. Med.*, 89, 640-644, 1977). Z vnosom farmacevtskega pripravka, ki vsebuje EPO, v organizem lahko pospešimo tvorbo novih eritrocitov. Farmacevtski pripravek se največ uporablja pri bolnikih z zmanjšano tvorbo EPO zaradi kroničnega obolenja ledvic, pri AIDS-u in rakavih bolnikih, ki se zdravijo s kemoterapijo in pri zdravljenju drugih vrst anemij (Danna s sod. v Erythropoietin in Clinical Applications - An International Perspective. New York, NY: Marcel Dekker; 301-324, 1990; Eschbach in sod., *N. England J. of Med.*,

316, 2, 73-78, 1987; Krane, *Henry Ford Hosp. Med. J.*, 31,3, 177-181, 1983). EPO, ki se uporablja v farmacevtskih preparatih, je rekombinantnega izvora in je produkt izražanja humanega gena za EPO v sesalskih celicah (EP 148605, EP 205564, EP255231). Poznani so tudi EPO analogi in EPO derivati, ki so med drugim opisani v: EP640619, EP 668351, WO 9412650, EP1064951, WO 0232957, WO 9533057, US 5916773, WO 09902710, US 5580853, US 5747446, US 5919758 in US 6107272.

Farmacevtski pripravki, ki vsebujejo humani serumski albumin, so med drugim opisani v patentih: EP 178665, EP 178576, US 5661125, WO 0061169. Humani serumski albumin lahko povzroča alergijske reakcije (Stafford CT in sod. *Ann Allergy*, 61(2), 85-88, 1988). Prav tako je njegova uporaba tvegana, kajti kljub testiranju krvi obstaja nevarnost za okužbo z virusi. Zato obstaja potreba po razvoju farmacevtskega pripravka, ki stabilizira EPO in hkrati ne vsebuje humanih proteinov.

V EP 306824, EP 607156, EP 528313, EP 528314 so opisani farmacevtski pripravki za EPO, ki kot stabilizator vsebujejo ureo.

V EP306824, EP 178665, GB 2171304, EP 528314, EP 528313 in EP 1002547 so opisani liofilizirani farmacevtski pripravki, ki vsebujejo EPO. Liofilizirani farmacevstki pripravki so v klinični uporabi manj praktični zaradi postopka rekonstitucije pred uporabo. Tak postopek je zamuden, obstaja tveganje za nepravilno uporabo ali nepravilno rekonstitucijo in običajno se liofiliziranim farmacevtskim pripravkom dodajajo dodatni stabilizatorji, ki so potrebni za ohranjanje aktivnosti proteina v postopku liofilizacije.

V US 5376632 je opisan farmacevtski pripravek, ki vsebuje alfa in beta ciklodekstrine.

V EP 607156, EP 528313 in EP178665 so opisani vodni farmacevtski pripravki, ki vsebujejo EPO in konzervanse, kot so benzil alkohol, klorbutanol, parabeni, fenoli in druge. Uporaba takih konzervansov lahko povzroča obarjanje EPO, kar klinično ni sprejemljivo.

V EP 909564, EP 528314, EP 430200 in WO 0061169 je opisana uporaba aminokislin in/ali kombinacija aminokislin in neionskih detergentov kot stabilizatorjev v farmacevtskih pripravkih, ki vsebujejo EPO.

V patentni prijavi WO 0187329 so opisani različni farmacevtski pripravki, ki so namenjeni stabilizaciji pegiliranega EPO analoga. Opisani farmacevtski pripavki temeljijo predvsem na uporabi sulfatnega pufra.

Farmacevtski pripravki za EPO, ki so opisani med drugim v RU 2128517, WO0061169, EP 528313, EP 607156, EP 528314, EP 178665, so pripravljeni v citratnem pufru. Citratni pufer povzroča bolečino na mestu aplikacije, zato je za klinično uporabo bolj primeren fosfatni pufer.

Opis slik

Slika 1: SDS-PAGE vzorcev od FP1 do FP8, z vsebnostjo EPO 10000 IU/ml, shranjenih pri 40°C (±2°C) 1 mesec (40). Kot pozitivno kontrolo (PK) za prisotnost EPO dimer smo uporabili EPO substanco v vodi, shranjeno pri 40°C (±2°C) 1 mesec. V vse žepke smo nanesli 0.4 μg substance.

Sestava farmacevtskih pripravkov FP1 do FP8:

FP1: polisorbat 80 (0,03% (masni/volumski (m/v))), glicin (0,5% (m/v)), fosfatni pufer 20 (mmol/l), NaCl (100 mmol/l)

FP2: glicin (0,5% (m/v)), glicerol (1,4% (m/v)) , fosfatni pufer (32 mmol/l)

FP3: glicin (0,5% (m/v)), Pluronic F68 (0,1% (m/v)), fosfatni pufer (20 mmol/l), NaCl (90,6 mmol/l)

FP4: sorbitol (4,5% (m/v)), Pluronic F68 (0,1% (m/v)), fosfatni pufer (20 mmol/l)

FP5: dekstran 70 (1% (m/v)), NaCl (123 mmol/l), fosfatni pufer (20 mmol/l)

FP6: glicerol (2% (m/v)), Pluronic F 68 (0,1% (m/v)), NaCl (17.1 mmol/l), fosfatni pufer (20 mmol/l)

FP7: glicerol (2% (m/v)), PVP K12 (0,5% (m/v)), fosfatni pufer (20 mmol/l).

FP8: PVP K12 (0.5% (m/v)), NaCl (123 mmol/l), fosfatni pufer (20 mmol/l)

Legenda:

Steza	**Vzorec**
1	prazna steza
2	Obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 μl
3	prazna steza
4	EPO-BRP (EPO standard Evropske farmakopeje)

5	FP1 40
6	FP2 40
7	FP3 40
8	FP4 40
9	FP5 40
10	FP6 40
11	FP7 40
12	FP8 40
13	PK
14	prazna steza
15	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl

Slika 2: SDS-PAGE vzorcev od FP1 do FP4, z vsebnostjo EPO 10000 IU/ml, shranjenih v hladilniku (HL) in shranjenih pri 40°C (±2°C) 1 mesec (40). Kot pozitivno kontrolo (PK) za prisotnost EPO dimer smo uporabili EPO substanco v vodi, shranjeno pri 40°C (±2°C) 1 mesec. V vse žepke smo nanesli 0.4 µg substance.

Legenda:

Steza	Vzorec
1	prazna steza
2	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl
3	prazna steza
4	EPO-BRP (EPO standard Evropske farmakopeje)
5	FP1 HL
6	FP2 HL
7	FP3 HL
8	FP4 HL
9	FP1 40
10	FP2 40
11	FP3 40
12	FP4 40
13	PK

14	prazna steza
15	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl

Slika 3: SDS-PAGE vzorcev od FP5 do FP8, z vsebnostjo EPO 10000 IU/ml, shranjenih v hladilniku (HL) in shranjenih pri 40°C (±2°C) 1 mesec (40). Kot pozitivno kontrolo (PK) za prisotnost EPO dimer smo uporabili EPO substanco v vodi, shranjeno pri 40°C (±2°C) 1 mesec. V vse žepke smo nanesli 0.4 µg substance.

Legenda:

Steza	Vzorec
1	prazna steza
2	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl
3	prazna steza
4	EPO-BRP (EPO standard Evropske farmakopeje)
5	FP5 HL
6	FP6 HL
7	FP7 HL
8	FP8 HL
9	FP5 40
10	FP6 40
11	FP7 40
12	FP8 40
13	PK
14	prazna steza
15	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl

Slika 4: SDS-PAGE vzorcev od FP1 do FP8, z vsebnostjo EPO 10000 IU/ml, shranjenih v hladilniku (HL) 10 tednov. Kot pozitivno kontrolo (PK) za prisotnost EPO dimer smo uporabili EPO substanco v vodi, shranjeno pri 40°C (±2°C) 1 mesec. V vse žepke smo nanesli 0.4 µg substance.

Legenda:

Steza	Vzorec

1	prazna steza
2	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl
3	prazna steza
4	EPO-BRP (EPO standard Evropske farmakopeje)
5	FP1 HL
6	FP2 HL
7	FP3 HL
8	FP4 HL
9	FP5 HL
10	FP6 HL
11	FP7 HL
12	FP8 HL
13	PK
14	prazna steza
15	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl

Slika 5: SDS-PAGE vzorcev od FP1 do FP8, z vsebnostjo EPO 10000 IU/ml, shranjenih pri 25°C (±2°C) 10 tednov (25). Kot pozitivno kontrolo (PK) za prisotnost EPO dimer smo uporabili EPO substanco v vodi, shranjeno pri 40°C (±2°C) 1 mesec. V vse žepke smo nanesli 0.4 µg substance.

Legenda:

Steza	Vzorec
1	prazna steza
2	obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl
3	prazna steza
4	EPO-BRP (EPO standard Evropske farmakopeje)
5	FP1 25
6	FP2 25
7	FP3 25
8	FP4 25
9	FP5 25

10 FP6 25
11 FP7 25
12 FP8 25
13 PK
14 prazna steza
15 obarvani standardi SDS-PAGE, Bio-Rad, nanos 4 µl

Slika 6: Relativni odziv EPO-ELISA (v %) vzorcev od FP1 do FP8, z vsebnostjo EPO 10000 IU/ml, shranjenih pri 40°C (±2°C) 1 mesec (40) glede na vzorce od FP1 do FP8, shranjene v hladilniku 1 mesec (HL)

Slika 7: Relativni odziv EPO-ELISA (v %) vzorcev od FP1 do FP8, z vsebnostjo EPO 10000 IU/ml, shranjenih pri 25°C (±2°C) 10 tednov (25) glede na vzorce od FP1 do FP8, shranjene v hladilniku 1 mesec (HL)

Opis izuma

V smislu izuma smo presenetljivo ugotovili, da tekoči farmacevtski pripravek, ki vsebuje PVP in ne vsebuje dodatkov živalskega ali humanega izvora, stabilizira EPO.

Farmacevtski pripravek, ki je predmet izuma, vsebuje naslednje komponente:

a. terapevtsko učinkovito količino EPO,
b. farmacevtsko sprejemljiv pufrski sistem in
c. PVP kot stabilizator

in ne vsebuje dodatkov živalskega ali/in humanega izvora.

Farmacevtski pripravek, ki je predmet izuma, opcijsko dodatno vsebuje:

d. sredstvo za izotonizacijo in/ali
e. eno ali več drugih farmacevtsko sprejemljivih pomožnih snovi.

Z izrazom eritropoietin (EPO) je mišljen protein, ki ima *in vivo* biološko aktivnost, da povzroči diferenciacijo in/ali proliferacijo zarodnih celic kostnega mozga do eritrocitov.

Z izrazom 'terapevtsko učinkovita količina EPO' je mišljena tista količina EPO, ki omogoča terapevtski učinek EPO.

Z izrazom 'stabilizator' je mišljena farmacevtsko sprejemljiva pomožna snov, ki stabilizira EPO.

Z izrazom ' stabilnost EPO' je mišljeno tako ohranjanje vsebnosti EPO kot tudi ohranjanje biološke aktivnosti EPO. K zmanjšanju stabilnosti EPO prispevajo med drugim naslednji procesi: adsorpcija EPO na stene ovojnine, denaturacija ali razgradnja EPO in tvorba agregatov, npr. EPO dimer in/ali EPO multimer in/ali sorodnih molekul z večjo molekulsko maso. Ti procesi so lahko posledica različnih dejavnikov, med drugim povišane temperature, neprimerne ovojnine, uporabe neprimernih stabilizatorjev EPO, sončne svetlobe, neprimernega postopka izdelave in/ali neprimernega postopka shranjevanja.

Farmacevtski pripravek, ki je predmet izuma, stabilizira EPO pri temperaturah, ki so višje od temperature hladilnika (2-8°C), posebno pri sobni temperaturi, pa tudi pri višjih temperaturah (na primer okoli 40°C).

Farmacevtski pripravek, ki je predmet izuma, vsebuje za stabilizacijo EPO le eno farmacevtsko sprejemljivo pomožno snov, PVP. Uporaba PVP tako nadomesti kombinacije različnih stabilizatorjev, ki so bili do sedaj uporabljeni v drugih farmacevtskih pripravkih, ki vsebujejo EPO. V primerjavi z uporabo dveh ali večih stabilizatorjev je uporaba enega stabilizatorja boljša v smislu ekonomičnosti priprave, manjših stroškov, pa tudi v smislu lažje in manj zamudne priprave farmacevtskega pripravka, pa tudi za bolnika v smislu vnosa manj dodatnih snovi v organizem.

V nekaterih znanih farmacevtskih pripravkih, ki vsebujejo EPO, se kot stabilizatorji uporabljajo neionski detergenti polisorbati (polisorbat 20, polisorbat 80...). V primerjavi s polisorbati, PVP omogoča uporabo gelske filtracije kot analizne metode za ugotavljanje vsebnosti EPO dimer, EPO multimer in drugih sorodnih molekul z višjo molekulsko maso, ki nastanejo kot posledica agregacije molekul EPO. Pri gelski kromatografiji se namreč polisorbati s podobno molsko maso eluirajo na istem mestu kot EPO. S tem v farmacevtskih pripravkih EPO, v katerih se kot stabilizatorji uporabljajo takšni polisorbati, za dokazovanje deleža EPO dimer ni mogoče uporabiti gelske kromatografije. Uporaba PVP tako prispeva k lažji

dokazljivosti (analizi) stabilnosti EPO, k večji varnosti in lažji kontroli kvalitete farmacevtskega pripravka, ki vsebuje EPO.

Farmacevtski pripravek, ki je predmet izuma, je tekoči farmacevtski pripravek in omogoča parenteralno aplikacijo in sicer subkutano, intravenozno ali intramuskularno aplikacijo, brez rekonstitucije, redčenja ali dodatnih predpriprav, ki bi lahko prispevale k zmanjšanju aktivnosti EPO kot tudi k dodatnim tehničnim težavam pri uporabi. Uporaba tekočega farmacevtskega pripravka je torej bolj praktična kot je uporaba liofiliziranih pripravkov, ki jih je potrebno pred uporabo rekonstituirati. Postopek liofilizacije večinoma zahteva prisotnost dodatnih stabilizatorjev, je energetsko zelo potraten in poveča stroške proizvodnje.

Farmacevtski pripravek, ki je predmet izuma, ne vsebuje humanih serumskih proteinov, pri katerih je mogoča okužba z virusi. Prav tako je s tem zmanjšana verjetnost za pojav raznih alergijskih reakcij, ki bi bile lahko posledica uporabe humanih serumskih albuminov. Pripravljen je v izotonični raztopini, ki je farmacevtsko sprejemljiva in ne povzroči stranskih učinkov.

Farmacevtski pripravek, ki je predmet izuma, se lahko uporablja za vse vrste EPO, med drugim za EPO alfa, EPO beta, EPO omega in različne druge profile EPO izoform, kot tudi za posamezne EPO izoforme, EPO analoge, izbrane iz skupine, ki obsega EPO dimere, NESP (hiperglikoziliran analog rekombinantnega humanega EPO), gensko-aktiviran EPO, pegiliran EPO, fuzijske proteine (oligomere in multimere) z EPO, hibridne molekule z EPO, fragmente EPO, homologe EPO, muteine EPO, EPO s spremenjenimi glikozilacijskimi profili. EPO je lahko pridobljen s tehnikami rekombinantne DNA tehnologije, npr. iz cDNA, genomske DNA ali sintetične DNA, lahko je naravnega izvora, pridobljen z izolacijskimi metodami, ali pa pridobljen z gensko aktivacijo, transgenimi metodami ali drugimi znanimi metodami.

V farmacevtskem pripravku, ki je predmet izuma, je terapevtsko učinkovita količina EPO izbrana v območju med 500 in 100000 IU/dozo ali več (1 IU ustreza približno 10 ng EPO), prednostno med 1000 in 40000 IU/dozo. V splošnem je sprejeto, da je učinkovita količina EPO od 1 do 500 IU/kg telesne teže, prednostno med 50 in 300 IU/kg telesne teže. Polnjen v farmacevtsko ovojnino, izbrano iz

skupine, ki zajema ampule, injekcijske brizge in viale. Te farmacevtske ovojnine omogočajo aplikacijo v območju volumna med 0.2 ml in 20 ml (doza).

Terapevstko učinkovita količina EPO nadalje zavisi od vrste in velikosti zdravljenega osebka, oblike in resnosti bolezenskega stanja in načina aplikacije.

Prednostno območje pH je med okoli 6 in okoli 8, bolj prednostno med 6.8 in 7.5 in najbolj prednostno okoli 7.0. Med pufrskimi sistemi se lahko uporablja vsak znani farmacevtsko sprejemljiv pufer, ki omogoča vzdrževanje pH v območju med okoli 6 in okoli 8, prednostno se uporablja fosfatni pufrski sistem, najbolj prednostno pufrski sistem: natrijev monobazični fosfat dihidrat/natrijev dibazični fosfat dihidrat ($NaH_2P0_4x2H_2O/Na_2HPO_4$ $x2H_2O$). Koncentracija fosfatnih soli je odvisna od pH in je izbrana v območju med 10 in 50 mM, prednostno med 15 in 35 mM, najbolj prednostno okoli 20 mM. Po potrebi pH uravnavamo s HCl, NaOH, citronsko kislino ali Na citratom.

Farmacevtski pripravek, ki je predmet izuma, vsebuje PVP kot stabilizator. Prednostna je uporaba nizkomolekularnega PVP (PVP K12 do K18), najbolj prednostna je uporaba PVP K12. Koncentracija PVP zajema območje med 0.01% in 1 %, bolj prednostno med 0.1 in 1.0%, najbolj prednostna je koncentracija 0.5% (m/v).

Farmacevtski pripravek, ki je predmet izuma, opcijsko dodatno vsebuje farmacevtsko sprejemljivo pomožno snov za vzdrževanje izotoničnosti raztopine. Ta pomožna snov je prednostno izbrana iz skupine anorganskih soli, prednostno $CaCl_2$ in NaCl, najbolj prednostno NaCl. Izbrana koncentracija izotoničnega sredstva je taka, da omogoča izotoničnost končnega tekočega farmacevtskega pripravka.

Farmacevtski pripravek opcijsko dodatno vsebuje enega ali več stabilizatorjev EPO, izbranih iz skupine, ki zajema površinsko aktivne snovi, kot so: glikol in glicerol estri, makrogol estri in etri, sorbitan derivati oz. polisorbati (polisorbat 20, polisorbat 80), poloksameri (Pluronic F68). Prednostno se uporablja Pluronic F68 v koncentraciji do 1%, najbolj prednostno Pluronic v koncentraciji 0.1%.

Za analizo farmacevtskega pripravka, ki je predmet izuma, smo uporabili naslednje metode: denaturirajočo analizo s poliakrilamidno gelsko elektroforezo

(SDS-PAGE) z imunodetekcijo, gelsko kromatografijo (SEC), EPO-ELISO ter *in vivo* testiranje na miškah.

SDS-PAGE z imunodetekcijo: Vzorci za nanos so bili pripravljeni v vzorčnem pufru brez reducenta. Uporabili smo vertikalno SDS-PAGE, gel NuPAGE Bis-Tris 12%, 8 x 8 cm, debeline 1.0 mm, 15 žepkov (Invitrogen) in MOPS SDS pufer za elektroforezo (Invitrogen). Elektroforezo je tekla 1 uro pri konstantni napetosti 200 V. Po elektro-prenosu proteinov iz gela na nitrocelulozno membrano je imuno-detekcija eritropoietina na membrani potekala v dveh stopnjah z uporabo primarnih protiteles (anti-huEpo, mišja, monoklonska) v prvi stopnji in sekundarnih protiteles (anti-mišja IgG, zajčja, poliklonska) konjugirana s hrenovo peroksidazo v drugi. Dodatek substrata za peroksidazo (4-kloro-1-naftol) sproži barvno encimsko reakcijo, katere netopen produkt tvori sivo-modre lise na mestih na membrani, kjer je vezan EPO.

SDS-PAGE z imunodetekcijo pokaže, da pri farmacevtskem pripravku, ki je predmet izuma (FP8), pri sobni temperaturi (slike 1-5) ne nastanejo EPO agregati, kot so npr. EPO dimere ali sorodne molekule z višjo molekulsko maso, pri povišani temperaturi pa nastajajo v manjši meri. Primerjava farmacevtskega pripravka, ki je predmet izuma, s farmacevtskim pripravkom FP1, ki vsebuje kot stabilizator kombinacijo polisorbatov in aminokisline glicina pri povišani temperaturi (1 mesec 40 °C) kaže (slike 1,2,3), da pri FP1 nastanejo EPO dimere. Nastanek EPO dimer je eden ključnih dejavnikov za zmanjšano stabilnost EPO. Prav tako je možno, da EPO agregati, npr. EPO dimere in sorodne molekule z večjo molekulsko maso, povzročijo neželjene stranske učinke po aplikaciji in s tem nelagodnosti pacienta, ki tak farmacevtski pripravek prejema. Možno je tudi, da taki agregati povzročijo imunski odziv organizma, kar prepreči nadaljnjo terapijo z EPO.

EPO-ELISA: Sistem EPO-ELISA Quantikine IVD, R&D Systems, za določanje koncentracije EPO temelji na uporabi dveh vrst protiteles ("sendvič-metoda"). Epo iz vzorca se veže na mišja monoklonska protitelesa, ki so imobilizirana na mikrotitrski ploščici in ki specifično prepoznajo humani eritropoietin. V drugi stopnji se na tako ujet EPO vežejo še zajčja poliklonska anti-EPO-protitelesa, ki so konjugirana s hrenovo peroksidazo. Dodatek kromogena (substrata za peroksidazo) sproži encimsko reakcijo, katere produkt je modro obarvan topen kompleks. Intenzivnost barve (spektrofotometrična meritev absorpcije) je premo-sorazmerna množini

konjugata vezanega na kompleks EPO-protitelo, ki je hkrati premo-sorazmerna vsebnosti EPO v preiskovanem vzorcu.

Primerjava farmacevtskega pripravka, ki je predmet izuma (FP8), z drugimi farmacevtskimi pripravki (FP1-FP7) (slika 6) kaže, da je pri FP8 adsorpcija EPO na stene vial pri povišani temperaturi (40°C 1 mesec) manjša ali enaka, pri sobni temperaturi pa primerljiva z drugimi pripravki (slika 7). Povečana adsorpcija na stene ovojnin prispeva k zmanjšanju stabilnosti EPO in s tem k nižji celokupni biološki aktivnosti EPO.

V nekaterih do sedaj znanih farmacevtskih pripravkih, ki vsebujejo EPO (glej stanje tehnike) so uporabljene aminokisline kot stabilizatorji. Vendar aminokisline ne delujejo vedno kot stabilizatorji EPO. Iz slik 6 in 7 je razvidno, da je stabilnost EPO v farmacevtskih pripravkih FP2 in FP3 (vsebujeta glicin) tako pri sobni kot pri povišani temperaturi (40°C 1 mesec) manjša v primerjavi z farmacevtskimi pripravki FP4, FP6 in FP8, ki ne vsebujejo glicina, pa tudi manjša od FP1, ki vsebuje glicin. Za doseganje ohranjanja stabilnosti farmacevtskega pripravka, ki vsebuje EPO, je torej potrebna pravilna kombinacija raznih stabilizatorjev, eksperimentalno je pa potrebno preveriti, katera kombinacija stabilizatorjev bo najbolj stabilizirala EPO. V predloženem izumu smo presenetljivo ugotovili, da PVP stabilizira EPO. Ugotovili smo tudi, da PVP v kombinaciji z glicerolom pri povišani temperaturi (40°C 1 mesec) ne stabilizira EPO (slika 6), medtem ko glicerol v kombinaciji s Pluronic F68 (sliki 6 in 7) stabilizira EPO tako pri sobni kot pri povišani temperaturi (40°C 1 mesec). EPO stabilizirajo le določene kombinacije farmacevtsko pomožnih snovi, ki pa niso predvidljive.

SEC: S SEC smo merili delež EPO dimer oz. sorodnih molekul z večjo molekulsko maso na vzorcih od FP1 do FP8 z vsebnostjo EPO 2000 IU/ml in 10000 IU/ml. Pri izvedbi smo uporabili limitni test po zahtevah Evropske farmakopeje (European Pharmacopeia 2002, 4. izdaja, Erythropoietin concentrated solution). Delež EPO dimer smo primerjali z redčeno raztopino vzorca (2%).

Vzorec	Ocena deleža dimer (približne vrednosti)	
	40°C 1 mesec	25°C 10 tednov
FP1 (2000 IU/ml)	*	*
FP1 (10000 IU/ml)	*	*
FP2 (2000 IU/ml)	<2% (pribl.1.3%)	/
FP2 (10000 IU/ml)	>2% (pribl. 2,2%)	/
FP3 (2000 IU/ml)	>2% (pribl. 3.7%)	/
FP3 (10000 IU/ml)	>2% (pribl. 4.3%)	/
FP4 (2000 IU/ml)	<2% (pribl, 0.3%)	/
FP4 (10000 IU/ml)	<2% (pribl. 1.2%)	/
FP5 (2000 IU/ml)	*	/
FP5 (10000 IU/ml)	>2% (pribl. 3.2%)	/
FP6 (2000 IU/ml)	<2% (pribl. 0,9%)	/
FP6 (10000 IU/ml)	>2% (pribl. 2,3%)	/
FP7(2000 IU/ml)	<2% (pribl. 0.4%)	/
FP7 (10000 IU/ml)	<2% (pribl. 1.5%)	/
FP8 (2000 IU/ml)	<2% (pribl. 0.2%)	/
FP8 (10000 IU/ml)	<2% (pribl. 1.6%)	/

** : deleža dimer ni bilo mogoče izračunati, ker komponente placeba motijo določitev*

/ : delež dimer je pod mejo detekcije

In vivo biološka aktivnost:

In vivo biološko aktivnost smo merili na vzorcu FP8 z vsebnostjo EPO 10000 IU/ml, shranjenem pri 25°C 10 tednov in v hladilniku 4 mesece. Biološko aktivnost smo merili s pomočjo *in vivo* metode na hipoksičnih miškah po postopkih iz Evropske farmakopeje. Izračun ocene biološke aktivnosti je bil prav tako izveden po priporočljivih postopkih iz Evropske farmakopeje (Eur. Pharmacopeia – 1997; Statistical Analysis of Results of Biological Assays and Tests; The parallel-line model). Po Evropski farmakopeji ocenjena vrednost biološke aktivnosti ne sme biti

manjša od 80% in ne večja od 120% označene aktivnosti. Cilj meritev biološke aktivnosti je torej doseganje območja med 80 in 120% vrednosti glede na začetno vrednost injeciranega EPO (10000 IU/ml) in dobljeni rezultat predstavlja oceno biološke aktivnosti in ne njene natančne vrednosti. Meja zaupanja ocenjene aktivnosti ne sme biti manjša od 64% in ne večja od 156% označene aktivnosti. Dobljeni rezultati so predstavljeni v tabeli:

Vzorec	Ocena biološke aktivnosti (80-120%)	Meja zaupanja (64-156%)
FP8 (25°C, 10 ted.)	9095 IU/ml (91%)	69-143%
FP8 (HL, 4 mes.)	9917 IU/ml (99%)	76-129%

Rezultati kažejo, da je ocenjena vrednost biološke aktivnosti znotraj zahtevanih meja in ustreza farmakopejskim zahtevam. Prav tako je meja zaupanja v zahtevanem območju.

Pogoji testiranja stabilnosti farmacevtskih pripravkov, ki vsebujejo EPO

HL-referenca	2 do 8 °C, hladilnik
40	40 °C ± 2°C, 75% rel.vl. ± 5%, klima komora
25	25 °C ± 2°C, 60% rel.vl. ± 5%, klimatiziran prostor

Predloženi izum prikazujejo, vendar v ničemer ne omejujejo naslednji primeri.

Primeri

Primera 1-2:Sestava farmacevtskih pripravkov (FP8), ki vsebujeta EPO

Opis pripravka	Aktivna učinkovina	Neaktivna dodana snov
FP8 (2000)		
	2000 IU EPO Lek	
		NaH_2P0_4 x2H_2O 1,164 mg
		Na_2HPO_4 x2H_2O 2,225 mg
		NaCl 7,200 mg
		PVP K12 5,000 mg
		NaOH za uravnavo pH (pH: 7.0 – 7.1)
		Voda do 1 ml

Opis pripravka	Aktivna učinkovina	Neaktivna dodana snov
FP8 (10000)		
	10000 IU EPO Lek	
		NaH_2P0_4 x2H_2O 1,164 mg
		Na_2HPO_4 x2H_2O 2,225 mg
		NaCl 7,200 mg
		PVP K12 5,000 mg
		NaOH za uravnavo pH (pH: 7.0 – 7.1)
		Voda do 1 ml

Kvalitete substanc:

Epoetin Lek kvalitete, ki jo določa Evropska farmakopeja (Ph Eur. kvaliteta),

Povidon K12 (poli[1-(2-okso-1-pirolidil)etilen], polividon, PVP) Ph Eur kvalitete, ki ustreza farmakopeji ZDA (USP kvalitete), dobavljen pri BASF, Ludwigshafen, Nemčija,

NaCl, Na_2HPO_4 x2H_2O, NaH_2P0_4 x2H_2O, NaOH, voda za injekcije so bile Ph. Eur. kvalitete.

Priprava farmacevtskih pripravkov, ki vsebujeta EPO

Placebo raztopino s PVP K12 smo pripravili tako, da smo v vodi za inj. pri sobni temperaturi raztopili med mešanjem na magnetnem mešalu najprej pufer (Na_2HPO_4 x2H_2O, NaH_2P0_4 x2H_2O), nato NaCl in stabilizator PVP K12. pH vrednost raztopine smo dvignili z 1M NaOH na 7,0 – 7,1. Dobili smo bistro brezbarvno raztopino.

Raztopino EPO Lek smo pripravili tako, da smo odtajano količino EPO Lek koncentrirane raztopine (preračunane na aktivnosti) pri sobni temperaturi dodali v placebo raztopino potem, ko smo iz iste raztopine enak volumen placebo raztopine odvzeli. Raztopino smo na magnetnem mešalu pri nizkih obratih premešali. Dobili smo bistro, brezbarvno raztopino.

Raztopine farmacevtskih pripravkov, ki vsebujejo EPO Lek v obeh koncentracijah, smo nato v aseptičnih pogojih v kvaliteti zraka klasa 100 sterilno filtrirali čez membranski filter s PVDF (Polyvinylidenfluorid) membrano z velikostjo por 0,2 μm in filtrirano raztopino napolnili po 0,8 ml v 2 ml-ske viale iz brezbarvnega cevnega stekla I. hidrolitske skupine, oprane in sterilizirane in jih zaprli s čepi iz brombutil kaučuka in z aluminijastimi zaporkami.

LEK farmacevtska družba d.d.

Patentni zahtevki

1. Stabilni farmacevtski pripravek, označen s tem, da vsebuje naslednje komponente:

a. terapevtsko učinkovito količino EPO
b. farmacevtsko sprejemljiv pufrski sistem
c. PVP

in opcijsko dodatno vsebuje

d. sredstvo za izotonizacijo in/ali
e. eno ali več farmacevtsko sprejemljivih pomožnih snovi

in ne vsebuje dodatkov živalskega in/ali humanega izvora.

2. Farmacevtski pripravek po zahtevku 1, označen s tem, da je farmacevtski pripravek tekoči.
3. Farmacevtski pripravek po zahtevku 2, označen s tem, da je količina EPO izbrana v območju med 500 in 100000 IU EPO na dozo.
4. Farmacevtski pripravek po zahtevku 3, označen s tem, da je količina EPO izbrana iz skupine, ki obsega: okoli 1000 IU/dozo, 2000 IU/dozo, okoli 3000 IU/dozo, okoli 4000 IU/dozo, okoli 10000 IU/dozo, okoli 20000 IU/dozo, okoli 25000 IU/dozo ali okoli 40000 IU/dozo.
5. Farmacevtski pripravek po zahtevkih od 1 do 4, označen s tem, da je pH raztopine izbran v območju med okoli 6 in okoli 8.
6. Farmacevtski pripravek po zahtevku 5, označen s tem, da je pH raztopine izbran v območju med 6.8 in 7.5.
7. Farmacevtski pripravek po zahtevku 6, označen s tem, da je izbran pH raztopine okoli 7.
8. Farmacevtski pripravek po zahtevkih od 1 do 7, označen s tem, da je izbrani pufrski sistem fosfatni pufer.
9. Farmacevtski pripravek po zahtevkih od 1 do 8, označen s tem, da je koncentracija PVP izbrana v območju med 0.01% in 1%.
10. Farmacevtski pripravek po zahtevku 9, označen s tem, da je koncentracija PVP izbrana v območju med 0.1% in 1%.

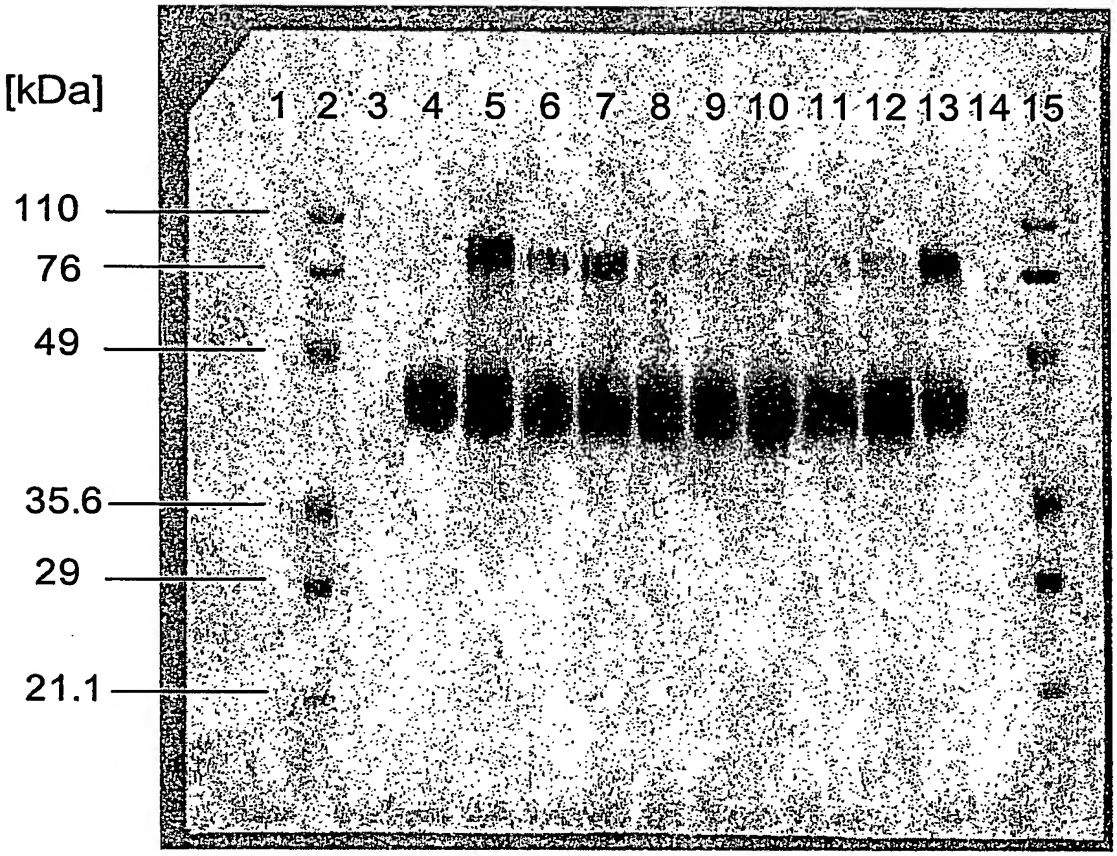
11. Farmacevtski pripravek po zahtevku 10, označen s tem, da je izbrana koncentracija PVP okoli 0.5%.
12. Farmacevtski pripravek po zahtevkih od 1 do 11, označen s tem, da je sredstvo za izotonizacijo izbrano iz skupine anorganskih soli.
13. Farmacevtski pripravek po zahtevku 12, označen s tem, da je izbrano sredstvo za izotonizacijo NaCl.
14. Postopek za pripravo farmacevtskega pripravka po zahtevkih od 1 do 13.
15. Uporaba farmacevtskega pripravka po zahtevkih od 1 do 14 za pripravo zdravil za zdravljenje bolezni, ki so indicirane za EPO.

LEK farmacevtska družba d.d.

Izvleček:

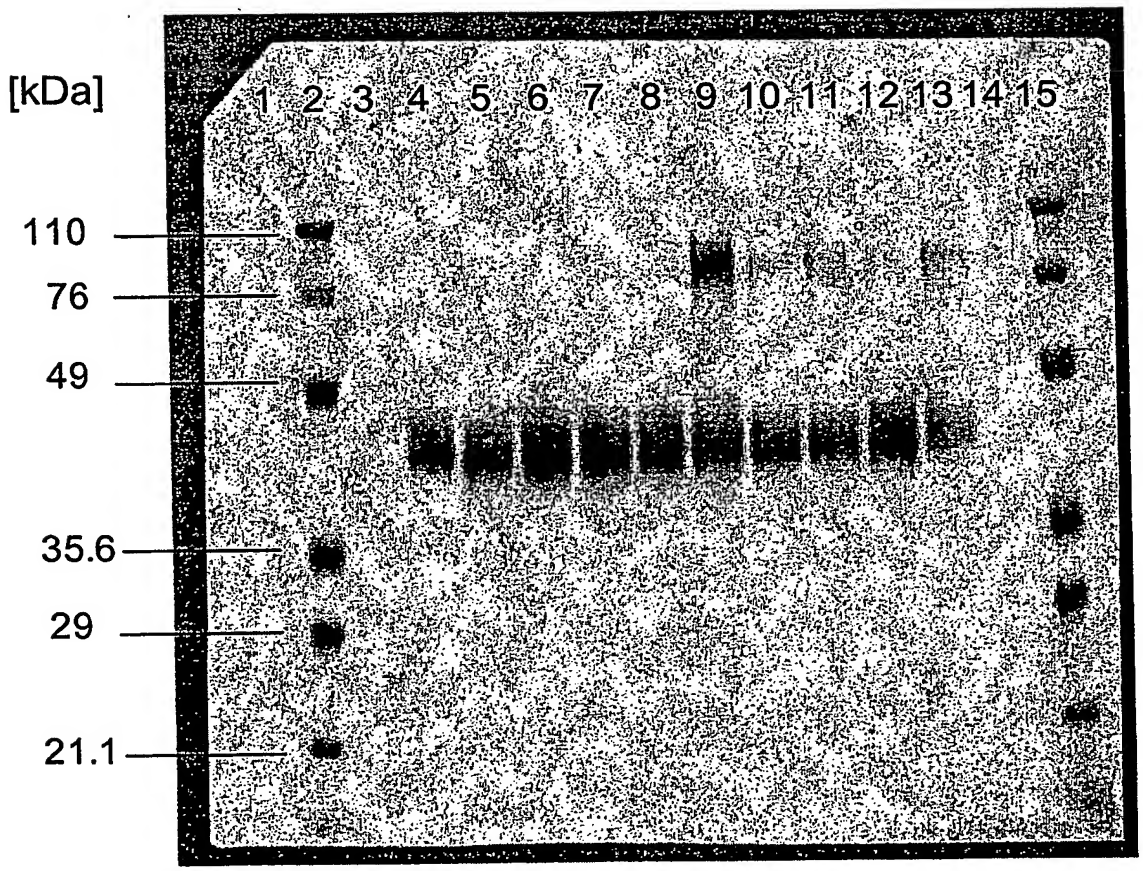
Izum se nanaša na nov stabilni tekoči farmacevtski pripravek za eritropoietin (EPO), ki stabilizira EPO in ne vsebuje dodatkov živalskega ali/in humanega izvora.

Slika 1



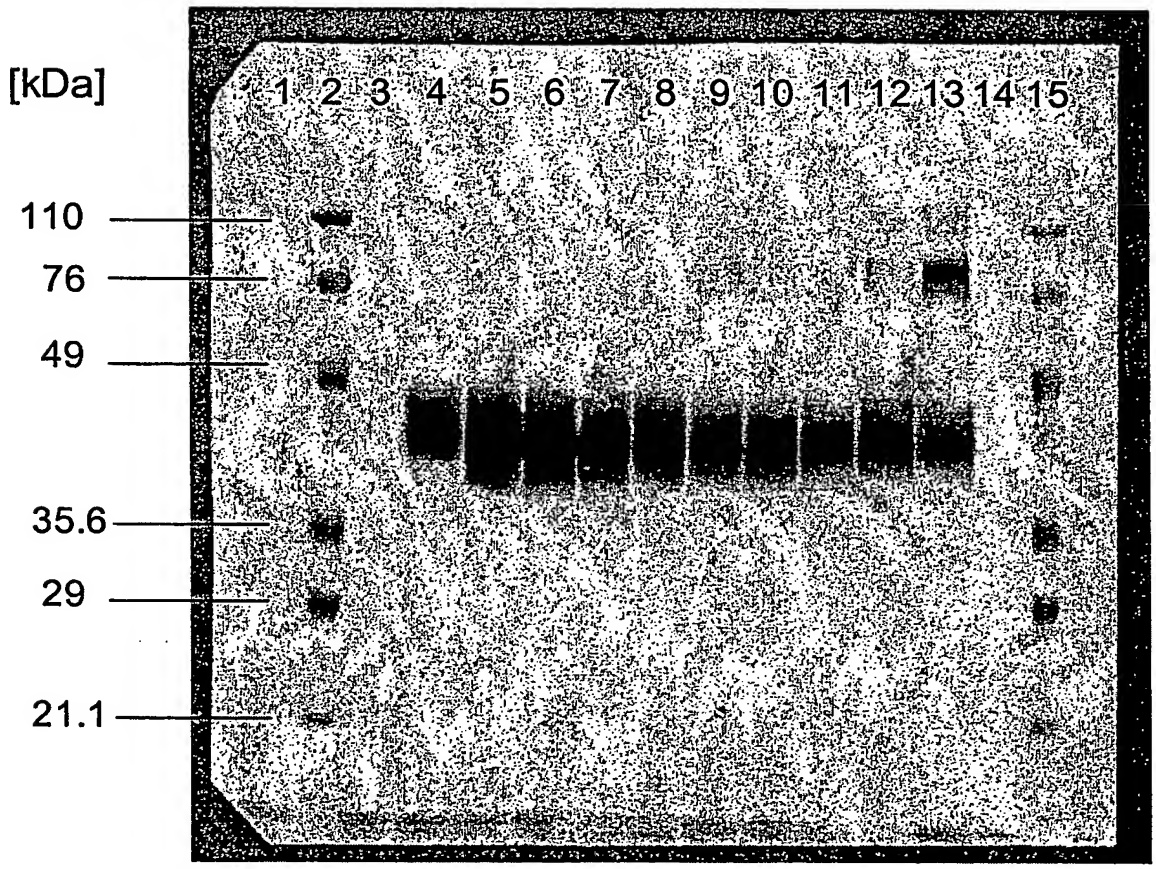
LEK farmacevtska družba d.d.

Slika 2



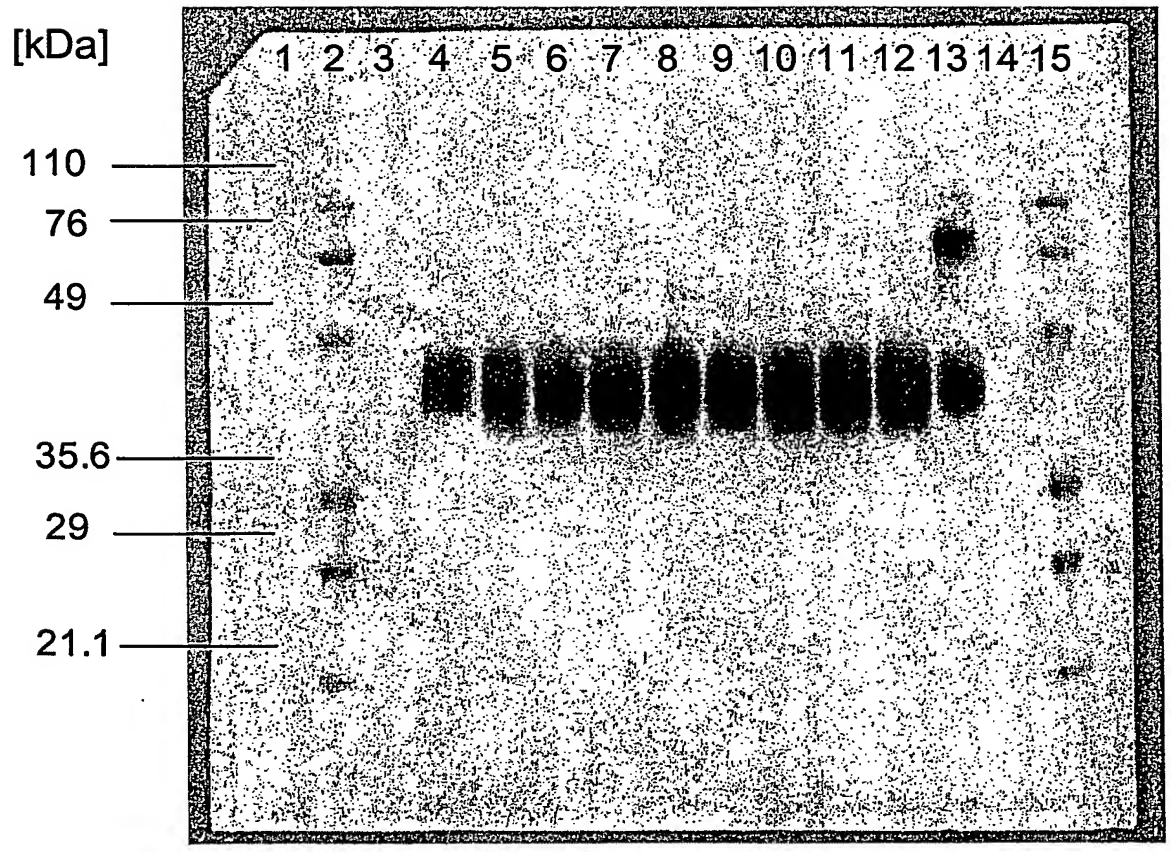
LEK farmacevtska družba d.d.

Slika 3



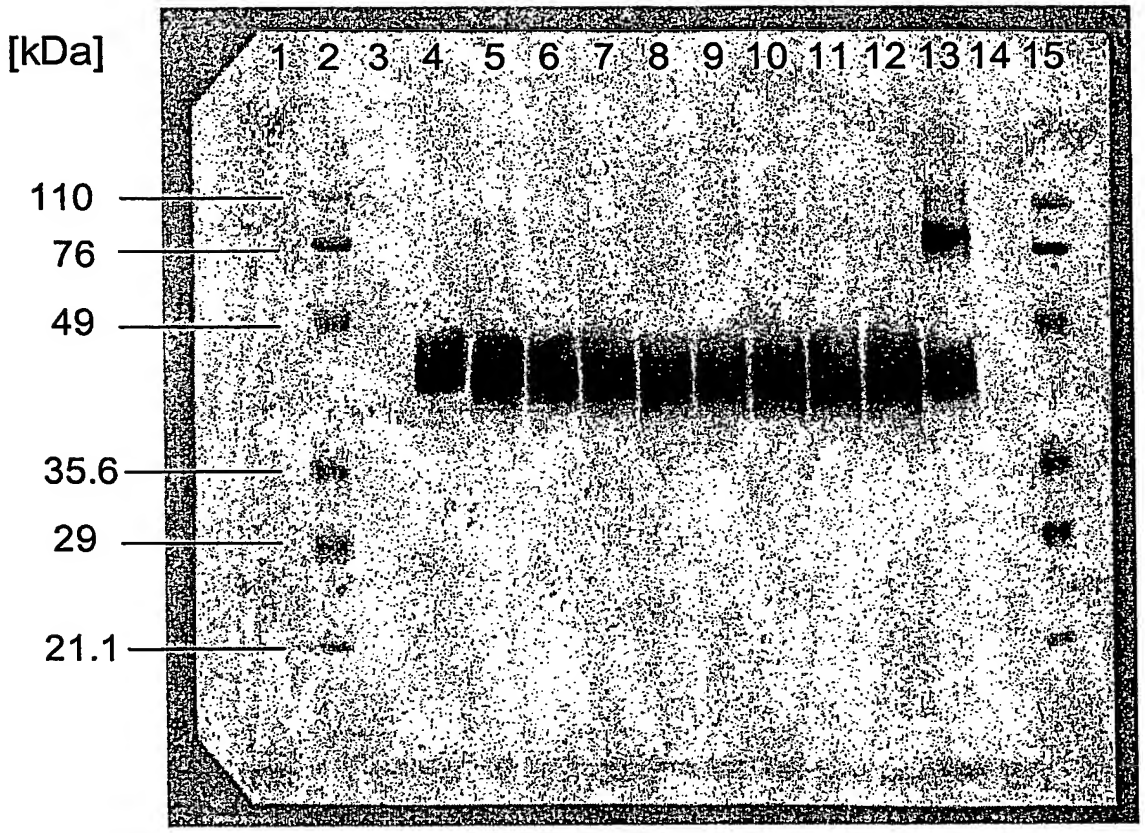
LEK farmacevtska družba d.d.

Slika 4



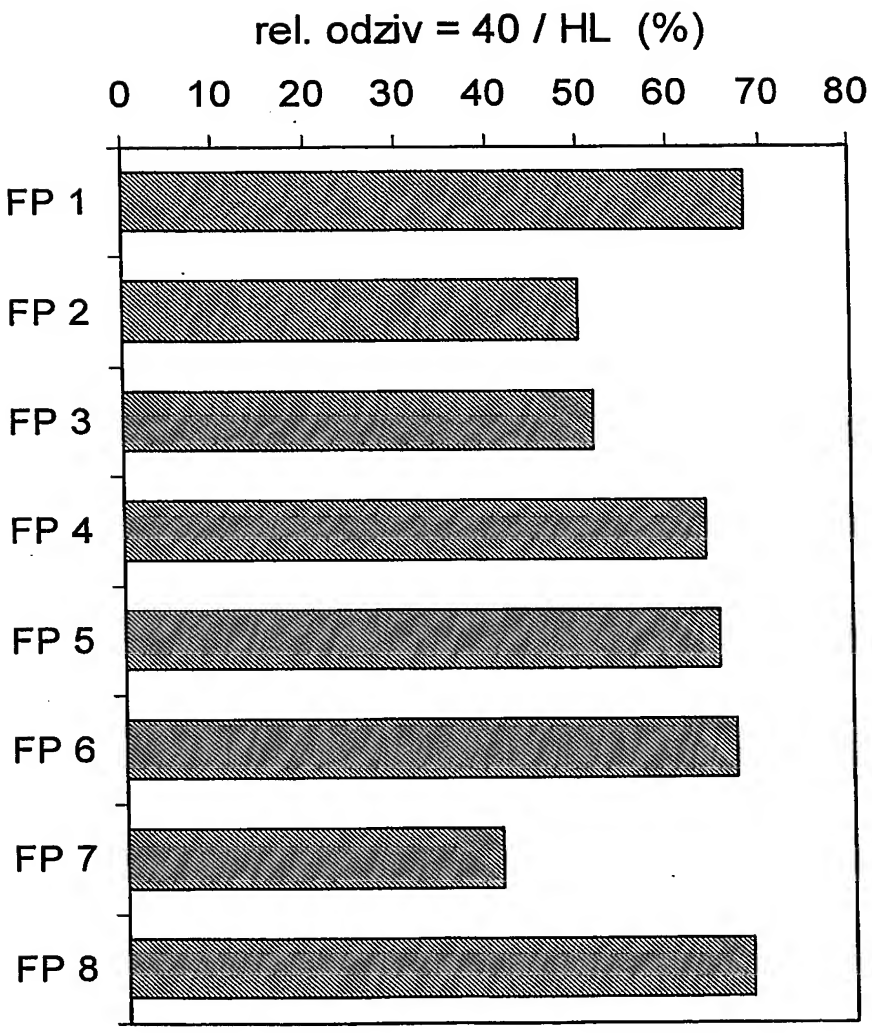
LEK farmacevtska družba d.d.

Slika 5



LEK farmacevtska družba d.d.

Slika 6



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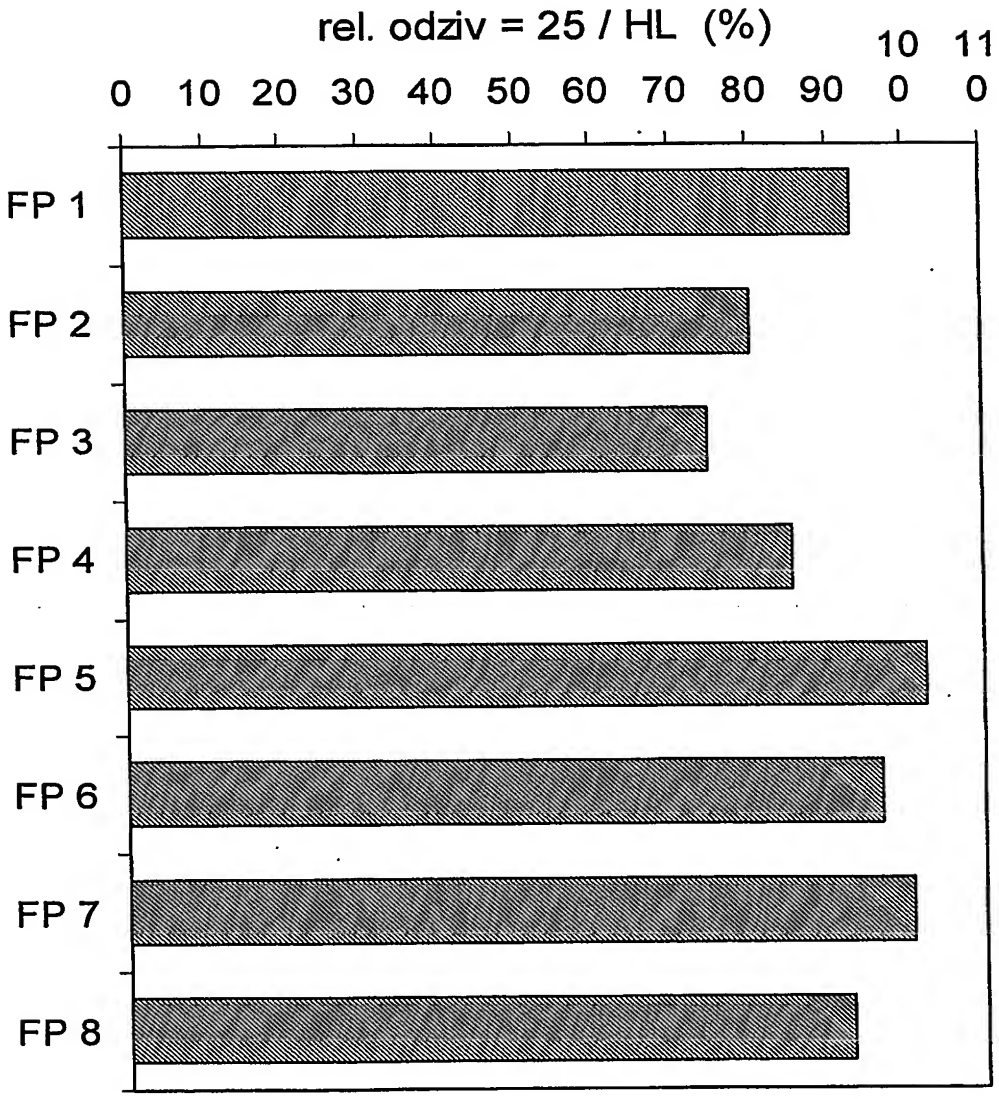
Slika 7



LEK farmacevtska družba d.d.

REPUBLIC OF SLOVENIA

Ministry of Economic Affairs

SLOVENIAN INTELLECTUAL PROPERTY OFFICE

Certificate

Slovenian Intellectual Property Office hereby certifies that the document annexed hereto is a true copy of the patent application, as follows:

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(21) Application No.: P-200200176

(54) Title: Stable pharmaceutical composition comprising erythropoietin

Ljubljana, October 10th, 2002

Janez Kukec-Mezek
Government Counsellor

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REPUBLIC OF SLOVENIA
MINISTRY OF SCEINCE AND TECHNOLOGY

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1. Address for correspondence: Lek Pharmaceuticals d.d. Intellectual Property Department Verovškova 57 1526 Ljubljana Slovenia Telephone: 580 20 05 Fax: 568 21 23 Code: bk/ 802	Request for grant of a patent Date of application receipt *(for official use only)* *17th July 2002* Application number *(for official use only)* **P-200200176**
2. Applicant (Family name followed by given name and address; for a legal entity, full official designation): Lek Pharmaceuticals d.d. Verovškova 57 1526 Ljubljana Slovenia	
3. Representative: Register number:	
4. Inventor (Family name followed by given name and address): Vukmirovič Andreja Dergomaška 16 1000 Ljubljana Slovenia	
5. Title of invention: Stable pharmaceutical composition comprising eryhtropoietin	
6. Claimed priority right:	
7. Additional requests: ☐ application for a shortened duration patent ☐ preliminary publication after the expiry of ___months ☐ application exempt form the Application number: ___: ___	
8. Statements: ☐ statement of common representative	

9. Enclosures:

- [x] Description of the invention, having 16 pages
- [x] Patent application (applications), having pages 2; number of claims: 15
- [x] Schemes (if required for patent description); number of sheets: 7
- [x] Abstract
- [ ] Voucher for the settlement of fees
- [ ] Declaration of depositing the microorganism (if it is a microbiological invention which cannot be described)
- [ ] Authorisation to the representative
- [ ] General authorisation to the represantative is deposed in the office under number: .....
- [ ] Declaration of priority right
- [x] Information of additional inventors
- [ ] Information of additional applicants
- [ ] Presentation of nucleotide or amino acid sequence in the description
- [ ] Application was previously faxed or mailed in electronic form
- [ ] _____

Košak Alenka

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Title of the invention

Stable pharmaceutical composition comprising erythropoietin

Field of the invention

The present invention relates to a new stable liquid pharmaceutical composition which comprises erythropoietin (EPO).

EPO is a glycoprotein hormone which regulates the formation of erythrocytes in mammals. It works as a growth and/or differential factor to the bone marrow progenitor cells and causes their proliferation and differentiation to erythrocytes.

The essential element of the present invention is a new stable liquid pharmaceutical composition comprising EPO, stabilises EPO and is free of additives of human or animal origin (e.g. serum proteins). The pharmaceutical composition is prepared in a pharmaceutically acceptable pH buffering system and comprises povidone (PVP) as a stabilising agent. The pharmaceutical composition optionally further comprises one or more pharmaceutically acceptable excipients. The pharmaceutical composition of the present invention is pharmaceutically acceptable for parenteral administration (e.g. for intramuscular, subcutaneous and/or intravenous administration) and is suitable for use in medicine.

Background of the invention

EPO acts as a growth and/or differential factor to the bone marrow progenitor cells and causes their differentiation to erythroblasts from which the erythrocytes are formed (Goldwasser et al, *J. Biol. Chem.,* 249, 4202-4211, 1974, Sherwood et al, *Endocrinology*, 103, 866-870, 1978). It is produced in adult kidneys (Sherwood et al, Endocrinology, 103, 866-870,1978) and in fetal liver (Zanjani et al, J.Lab. clin. Med., 89, 640-644, 1977).

The administration of pharmaceutical composition of EPO into the human body accelerates the production of erythrocytes. The pharmaceutical composition of EPO is mostly used in the treatment of patients with decreased production of EPO due to chronic renal failure and AIDS, in cancer patients with chemotherapy

treatment and in the treatment of various anemias (Danna et al, in Erythropoietin in Clinical Applications - An International Perspective. New York, NY: Marcel Dekker; 301-324, 1990; Eschbach et al, *N. England J. of Med.*, 316, 2, 73-78, 1987; Krane, *Henry Ford Hosp. Med. J.*, 31,3, 177-181, 1983).

The recombinant EPO which is used in pharmaceutical compositions is the product of expression of human EPO gene in mammalian cells (EP 148605, EP 205564, EP255231). There are also some EPO analogs and derivatives known and are described in: EP640619, EP 668351, WO 9412650, EP1064951, WO 0232957, WO 9533057, US 5916773, WO 09902710, US 5580853, US 5747446, US 5919758 in US 6107272.

Pharmaceutical compositions of EPO which comprise human serum albumin are among others described in: EP 178665, EP 178576, US 5661125, WO 0061169. Human serum albumin can cause allergic reactions (Stafford CT et al,. *Ann Allergy*, 61(2), 85-88, 1988). Furthermore, their use presents a risk of infection with viruses despite the blood screening. Therefore, pharmaceutical compositions of EPO that stabilise EPO and are free of human proteins are needed.

In EP 306824, EP 607156, EP 528313, EP 528314 the pharmaceutical compositions in which urea is used as EPO stabilising agent are described.

EP306824, EP 178665, GB 2171304, EP 528314, EP 528313 and EP 1002547 describe lyophilised compositions which comprise EPO. The lyophilised pharmaceutical compositions are less practical in clinical use because the end user must reconstitute the composition prior to administration. The reconstitution process is time-consuming, poses risks of improper handling or may be reconstituted improperly and certain additives such as stabilisers are usually required to retain sufficient activity of the protein during the process of lyophilisation.

US 5376632 describes a pharmaceutical composition, in which alpha and beta cyclodextrins are used.

EP 607156, EP 528313 and EP178665 describe aqueous pharmaceutical compositions of EPO which comprise EPO and antimicrobial preservatives such as benzyl alcohol, parabens, phenols, and others. The precipitation of EPO was observed in these pharmaceutical compositions that is not clinically acceptable.

EP 909564, EP 528314, EP 430200 and WO 0061169 describe the use of amino acids and/or the combination of amino acids and non-ionic detergents as stabilising agents in the pharmaceutical compositions which comprise EPO.

Patent application WO 0187329 describes different pharmaceutical compositions of pegylated EPO analog. The described pharmaceutical compositions are essentially based on the use of sulphate buffer.

Pharmaceutical compositions of EPO which are described among others in: RU 2128517, WO0061169, EP 528313, EP 607156, EP 528314, EP 178665, are prepared in citrate buffer. The citrate buffer causes pain at the injection site, therefore, the phosphate buffer is more preferable for clinical use.

Description of drawings

Figure 1: SDS-PAGE of the samples from FP1 to FP8, with EPO content of 10000 IU/ml, stored at 40°C (±2°C) 1 month (40). EPO substance in water stored at 40°C (±2°C) 1 month was taken as a positive control (PK) for the determination of the content of EPO dimers. 0.4 μg was loaded in each lane.

The composition of the pharmaceutical compositions from FP1 to FP8:

FP1: polysorbate 80 (0.03% (weight/volume (w/v))), glycine (0.5% (w/v)), phosphate buffer 20 (mmol/l), NaCl (100 mmol/l)

FP2: glycine (0.5% (w/v)), glycerol (1.4% (w/v)) , phosphate buffer (32 mmol/l)

FP3: glycine (0.5% (w/v)), Pluronic F68 (0.1% (w/v)), phosphate buffer (20 mmol/l), NaCl (90.6 mmol/l)

FP4: sorbitol (4.5% (w/v)), Pluronic F68 (0.1% (w/v)), phosphate buffer (20 mmol/l)

FP5: dextran 70 (1% (w/v)), NaCl (123 mmol/l)

FP6: glycerol (2% (w/v)), Pluronic F 68 (0,1% (w/v)), NaCl (17.1 mmol/l) phosphate buffer (20 mmol/l)

FP7: glycerol (2% (w/v)), PVP K12 (0.5% (w/v)), phosphate buffer (20 mmol/l).

FP8: PVP K12 (0.5% (w/v)), NaCl (123 mmol/l), phosphate buffer (20 mmol/l)

Legend:

Lane	Sample
1	empty lane
2	prestained SDS-PAGE standards, Bio-Rad, 4 µl load
3	empty lane
4	EPO-BRP (EPO standard of the European Pharmacopoeia)
5	FP 1 40
6	FP2 40
7	FP3 40
8	FP4 40
9	FP5 40
10	FP6 40
11	FP7 40
12	FP8 40
13	PK
14	empty lane
15	prestained SDS-PAGE standards, Bio-Rad, 4 µl load

Figure 2: SDS-PAGE of the samples from FP1 to FP4, with EPO content of 10000 IU/ml, stored in the refrigerator (HL) and stored at 40°C (±2°C) 1 month (40). EPO substance in water stored at 40°C (±2°C) 1 month was taken as a positive control (PK) for the determination of the content of EPO dimers. 0.4 µg was loaded in each lane.

Legend:

Lane	Sample
1	empty lane
2	prestained SDS-PAGE standards, Bio-Rad, 4 µl load
3	empty lane
4	EPO-BRP (EPO standard of the European Pharmacopoeia)
5	FP 1 HL
6	FP2 HL
7	FP3 HL
8	FP4 HL
9	FP1 40
10	FP2 40
11	FP3 40
12	FP4 40
13	PK
14	empty lane
15	prestained SDS-PAGE standards, Bio-Rad, 4 µl load

Figure 3: SDS-PAGE of the samples from FP5 to FP8, with EPO content of 10000 IU/ml, stored in the refrigerator (HL) and stored at 40°C (±2°C) 1 month (40). EPO substance in water stored at 40 °C (±2°C) 1 month was taken as a positive control (PK) for the determination of the content of EPO dimers. 0.4 µg was loaded in each lane.

Legend:

Lane	Sample
1	empty lane
2	prestained SDS-PAGE standards, Bio-Rad, 4 µl load
3	empty lane
4	EPO-BRP (EPO standard of the European Pharmacopoeia)
5	FP 5 HL
6	FP6 HL
7	FP7 HL
8	FP8 HL
9	FP5 40
10	FP6 40
11	FP7 40
12	FP8 40
13	PK
14	empty lane
15	prestained SDS-PAGE standards, Bio-Rad, 4 µl load

Figure 4: SDS-PAGE of the samples from FP1 to FP8, with EPO content of 10000 IU/ml, stored in the refrigerator (HL) 10 weeks. EPO substance in water stored at 40°C (±2°C) 1 month was taken as a positive control (PK) for the determination of the content of EPO dimers. 0.4 µg was loaded in each lane.

Legend:

Lane	Sample
1	empty lane
2	prestained SDS-PAGE standards, Bio-Rad, 4 µl load
3	empty lane
4	EPO-BRP (EPO standard of the European Pharmacopoeia)
5	FP1 HL
6	FP2 HL
7	FP3 HL
8	FP4 HL
9	FP5 HL
10	FP6 HL
11	FP7 HL
12	FP8 HL
13	PK
14	empty lane
15	prestained SDS-PAGE standards, Bio-Rad, 4 µl load

Figure 5: SDS-PAGE of the samples from FP1 to FP8, with EPO content of 10000 IU/ml, stored at 25°C (±2°C) 10 weeks (25). EPO substance in water stored at 40°C (±2°C) 1 month was taken as a positive control (PK) for the determination of the content of EPO dimers. 0.4 µg was loaded in each lane.

Legend:

Lane	Sample
1	empty lane
2	prestained SDS-PAGE standards, Bio-Rad, 4 µl load
3	empty lane
4	EPO-BRP (EPO standard of the European Pharmacopoeia)
5	FP1 25
6	FP2 25
7	FP3 25
8	FP4 25
9	FP5 25
10	FP6 25
11	FP7 25
12	FP8 25
13	PK
14	empty lane
15	prestained SDS-PAGE standards, Bio-Rad, 4 µl load

Figure 6: Relative response EPO-ELISA (in %) of the samples from FP1 to FP8, with the EPO content of EPO 10,000 IU/ml, stored at 40°C (±2°C) 1 month (40) to the samples from FP1 to FP8, stored in the refrigerator for 1 month (HL)

Figure 7: Relative response EPO-ELISA (in %) of the samples from FP1 to FP8, with the EPO content of EPO 10,000 IU/ml, stored at 25°C (±2°C) for 10 weeks (25) to the samples from FP1 to FP8, stored in the refrigerator for 10 weeks (HL).

Description of the invention

Surprisingly, it was found that a liquid pharmaceutical composition comprising PVP and being free of human and/or animal additives stabilised EPO.

The pharmaceutical composition of the present invention comprises the following components:

a. a therapeutically effective amount of EPO,

b. a pharmaceutically acceptable pH buffering system and

c. PVP as a stabiliser

and is free additives of human and/or animal origin.

The pharmaceutical composition of the present invention optionally further comprises:

d. an isotonifying agent and/or

e. one or more other pharmaceutically acceptable excipients.

The term 'erythropoietin (EPO)' refers to a protein with the *in vivo* biological activity of causing the differentiation and/or proliferation of bone marrow progenitor cells to erythrocytes.

The term 'therapeutically effective amount of EPO' refers to the amount of EPO which enables the therapeutical effect of EPO.

The term 'stabiliser' refers to a pharmaceutical acceptable excipient which stabilises EPO.

The term 'EPO stability' refers as to the maintenance of EPO content as well as to the maintenance of EPO biological activity. The EPO stability can be decreased by the following processes: adsorption of EPO to the vial walls, denaturation or degradation of EPO and aggregate formation, *e. g.* EPO dimers and/or EPO multimers and /or similar molecules with higher molecular weight. These processes occur due to exposing of the samples to different conditions, e.g. higher temperature, inappropriate vials, use of wrong stabilisers of EPO, sunshine, improper way of storing and/or improper storing.

The pharmaceutical composition of the present invention stabilises EPO at temperatures above refrigerator temperature (2-8°C), especially at room temperature and even at higher temperatures (for example at about 40°C).

In the pharmaceutical composition of the present invention only PVP is used as the EPO stabiliser. The use of PVP can therefore replace the combinations of different stabilisers which are known to be used in other pharmaceutical compositions of EPO described in the prior art. The preparation of pharmaceutical composition which comprises only one stabiliser instead of two or more stabilisers is better from the economical viewpoint. When compared with the use of two or more

stabilisers, the use of one stabiliser is better from the economical viewpoint, lower expenses and also the preparation is more easily performed, less time consuming and the patient receives less additives in the body.

In some known pharmaceutical compositions which comprise EPO the non-ionic detergents polysorbates (Polysorbate 20, Polysorbate 80...) are used as stabilisers of EPO. The use of PVP is advantageous over the use of polysorbates because gel filtration can be used as analytical method for the determination of EPO dimers, EPO multimers and other molecules with higher molecular weight resulting from the aggregation of EPO molecules. The polysorbates are eluted at the same time as are EPO dimers by gel filtration. Therefore, gel filtration cannot be used as a detection method for EPO dimers for the pharmaceutical compositions comprising polysorbates. The use of PVP therefore contributes to easier proving of EPO stability, increased safety and easier control of the quality of pharmaceutical composition which comprises EPO.

The pharmaceutical composition of the present invention is a liquid pharmaceutical composition which enables the parenteral administration, i.e. subcutaneous, intravenous or intramuscular administration, without reconstitution, dilution or additional preparation steps which may contribute both to lowering of EPO activity and additional technical problems occurring at administration. The use of a liquid pharmaceutical composition is therefore more practical than the use of lyophilised compositions. The reconstitution process requires the presence of additional stabilisers, is energy consuming and increases the costs of production.

The pharmaceutical composition of the present invention is free of human serum proteins which pose a risk of infection with viruses. Furthermore the probability of occurrence of different allergic reactions that may be caused by human serum albumin is diminished. It is prepared in isotonic solution, is pharmaceutically acceptable and does not cause side effects.

The pharmaceutical composition of the present invention can be used for all forms of EPO, comprising EPO alpha, EPO beta, EPO omega and other different EPO isoform profiles as well as for specific single EPO isoforms, EPO analogs selected from the group which comprises EPO dimers, NESP (hyperglycosylated

analog of recombinant human EPO), gene activated EPO, pegylated EPO, fusion protein (oligomers and multimers) with EPO, hybrid molecules with EPO, EPO fragments, EPO homologues, EPO muteins, EPO with modified glycosylation profiles. EPO can be produced by using recombinant DNA technology, for example, from cDNA, genomic DNA or synthetic DNA, can be of natural origin produced by isolation methods or produced by gene activated, transgenic or other known methods.

The pharmaceutical composition of the present invention comprises from 500 to 100,000 units or more EPO per dose (1 IU corresponds to about 10 nanograms of recombinant EPO), preferably from 100 to 40,000 IU per dose. In general, it is contemplated that an effective amount will be from 1 to 500 IU/kg body weight and more preferably from 50 to 300 IU/kg body weight especially EPO given subcutaneously. The effective amount will further depend on the species and size of the subject being treated, the particular condition or disease being treated and its severity and the route of administration. It can be filled in pharmaceutical package selected from the group which comprises ampoules, injection syringes and vials. These pharmaceutical packages enable the administration of the volumes in the range from 0.2 to 20 ml (dose). The therapeutically effective amount of EPO further depends on the species and size of the subject being treated, the particular condition or disease being treated and its severity and the route of administration.

The preferred pH range is between about 6 and about 8 with about 6.8 and 7.5 being more preferred, and a pH of about 7.0 being most preferred. Among buffer systems every known pharmaceutically acceptable buffer which is capable of maintaining pH in the range from about 6 to about 8 can be used. The use of a phosphate buffer system, especially sodium phosphate dibasic and sodium phosphate monobasic ($NaH_2P0_4x2H_2O/Na_2HPO_4$ $x2H_2O$) is preferred.

The concentration of phosphate salts depends on the desired pH of the composition. The preferred concentration is in the range between 10 and 50 mM, more preferred between 15 and 35 mM, most preferred about 20 mM. If needed, pH can be adjusted with HCl, NaOH, citric acid or sodium citrate.

The pharmaceutical composition of the present invention comprises PVP as a stabiliser. The use of low molecular weight PVP (PVP K12 to K18) is preferred, the

use of PVP K12 is mostly preferred. The concentration of PVP is in the range between about 0.01% and 1.0%, more preferred between 0.1 and 1.0%, most preferred of about 0.5% (w/v).

The pharmaceutical composition of the present invention optionally further comprises a pharmaceutically acceptable excipient capable of rendering the compositions of the present invention isoosmotic with human blood. This excipient is selected from the group of inorganic salts, preferably $CaCl_2$ and NaCl, most preferably NaCl. The concentration of the isotonifying agent enables the isotonicity of the final liquid pharmaceutical composition.

The pharmaceutical composition of the present invention optionally further comprises one or more EPO stabilisers selected from the group which comprises surface active agents such as glycol and glycerol esters, macrogel esters and ethers and sorbitan derivatives/polysorbates (e.g. Polysorbate 20, Polysorbate 80), poloxameres (Pluronic F68). More preferred is Pluronic F68 in the concentration less than 1% or about 1%, most preferred in the concentration of 0.1%.

The following analytical methods were used for the analysis of the pharmaceutical composition of the present invention: SDS-PAGE with immunodetection, size exclusion chromatography (SEC), EPO-ELISA and *in vivo* biological activity assay on mice.

SDS-PAGE with immunodetection: The loading samples were prepared in the loading buffer free of a reducing agent. The vertical SDS-PAGE was used: gel NuPAGE Bis-Tris 12%, 8 x 8 cm, thickness 1.0 mm, 15 lanes (Invitrogen) in MOPS SDS electrophoresis buffer (Invitrogen). Electrophoresis ran 1 hour at constant voltage of 200 V. After the electro-transfer of the proteins from the gel to the nitro-cellulose membrane, the immunodetection was performed in two steps. In the first step the primary antibodies (anti-huEPO, mouse, monoclonal) were used. In the second step the secondary antibodies (anti-mouse IgG, rabbit, polyclonal) conjugated to horseradish peroxidase were used. The addition of the peroxidase substrate (4-chloro-1naphtol) triggers colour enzyme reaction and the insoluble product formed grey-blue spots on the membrane parts where EPO was bound.

SDS-PAGE with immunodetection shows that EPO aggregates for example EPO dimers and similar molecules with high molecular weight do not occur in the

pharmaceutical composition of the present invention (FP8) at room temperature (Figures 1-5). At elevated temperature they are present in small amounts. The comparison of EPO stability at elevated temperature (1 month at 40°C) of the pharmaceutical composition of the present invention with the pharmaceutical composition FP1, in which the combination of polysorbates and amino acid glycine is used (Figures 1,2,3), shows that EPO dimers are formed in FP1. The formation of EPO dimers is one of the crucial factors for decreased EPO stability. It is also possible that EPO aggregates, *e. g.* EPO dimers and similar molecules with higher molecular weight cause undesired side effects after the application and discomfort of the patient being treated with such a pharmaceutical composition. It is also possible that these aggregates cause the body's immune response and the treatment with EPO has to be stopped.

EPO-ELISA: System EPO-ELISA Quantikine IVD, R&D Systems for the determination of EPO concentration, is based on the use of two kinds of antibodies ("sandwich method"). EPO from the sample binds to the mouse monoclonal antibodies which are immobilised to the microtitre plate and which specifically recognise human EPO. In the second step the rabbit polyclonal anti-EPO antibodies conjugated with horseradish peroxidase are bound to the immobilised EPO. The addition of a chromogen (peroxidase substrate) triggers the enzyme reaction and a blue coloured complex is formed. The amount of colour generated (spectrophotometrical measurement of absorption) is directly proportional to the amount of conjugate bound to the EPO antibody complex which, in turn, is directly proportional to the amount of EPO in the tested sample.

Comparison of the pharmaceutical composition of the present invention (FP8) with other prepared pharmaceutical compositions (FP1-FP7) (Figure 6) shows that in FP8 the adsorption of EPO to the vial walls at elevated temperature (40°C 1 month) is lower than or equal to the adsorption of EPO to the vial walls in other compositions, and at room temperature the adsorption of EPO to the vial walls is comparable in FP8 and other compositions (Figure 7). The increased adsorption to the vial walls decreases the EPO stability and the entire biological activity is decreased.

Amino acids have been used as stabilising agents of EPO in the compositions described in prior art (see Background of the invention). But amino acids do not always exhibit a stabilising effect on EPO. In Figures 6 and 7, it is seen that at elevated temperatures (40°C 1 month) the stability of pharmaceutical compositions FP2 and FP3 (comprising glycine) is lower than the stability of pharmaceutical preparations FP4, FP6 and FP8 not containing glycine and is also lower than FP1 comprising glycine. High EPO stability can be obtained with the use of the right combination of different stabilising agents, but their composition cannot be predicted and can only be experimentally determined. In the pharmaceutical composition of the present invention it was surprisingly found that PVP stabilised EPO. It was also found that PVP with the combination with glycerol did not act as an EPO stabilising agent at elevated temperature (40°C 1 month) (Figure 6), glycerol in the combination of Pluronic F68, on the other hand, had EPO stabilising effect at room and elevated temperatures (40°C 1 month) (Figures 6 and 7). Stability of EPO, therefore, depends on the combinations of different pharmaceutically acceptable excipients which are not predictable.

SEC: SEC was used to determine the proportion of EPO dimers or similar molecules with higher molecular weight in the samples from FP1 to FP8 with the EPO content from 2000 IU/ml to 10000 IU/ml. The limit assay following the protocols of the European Pharmacopoeia was used (European Pharmacopoeia 2002, 4th edition, Erythropoietin concentrated solution). The proportion of EPO dimers was compared with the diluted solution of the samples (2%).

Sample	The estimation of EPO dimer proportions (approximate values)	
	40°C 1 month	25°C 10 weeks
FP1 (2000 IU/ml)	*	*
FP1 (10000 IU/ml)	*	*
FP2 (2000 IU/ml)	<2% (approx. 1.3%)	/
FP2 (10000 IU/ml)	>2% (approx. 2.2%)	/
FP3 (2000 IU/ml)	>2% (approx. 3.7%)	/

FP3 (10000 IU/ml)	>2% (approx. 4.3%)	/
FP4 (2000 IU/ml)	<2% (approx. 0.3%)	/
FP4 (10000 IU/ml)	<2% (approx. 1.2%)	/
FP5 (2000 IU/ml)	*	/
FP5 (10000 IU/ml)	>2% (approx. 3.2%)	/
FP6 (2000 IU/ml)	<2% (approx. 0.9%)	/
FP6 (10000 IU/ml)	>2% (approx. 2.3%)	/
FP7(2000 IU/ml)	<2% (approx. 0.4%)	/
FP7 (10000 IU/ml)	<2% (approx. 1.5%)	/
FP8 (2000 IU/ml)	<2% (approx. 0.2%)	/
FP8 (10000 IU/ml)	<2% (approx. 1.6%)	/

** : the determination of the proportion of dimers was not possible due to polysorbates from placebo*

/ :dimers under detection limit

In vivo biological activity:

In vivo biological activity was measured in the sample FP8 with EPO content of 10,000 IU/ml, stored at 25°C for 10 weeks and in the refrigerator for 4 months. The protocol for *in vivo* determination of biological activity on hypoxic mice described in Eur. Ph. was used. The estimation of biological activity was performed under the protocols from Eur. Ph. as well (Eur. Pharmacopoeia – 1997; Statistical Analysis of Results of Biological Assays and Tests; The parallel-line model). Under the demands of Eur. Ph the estimated value of biological activity should be in the range between 80% and 120% of the marked activity. The aim of the method is to reach the range between 80% and 120% regarding the content of the EPO injected (10,000 IU/ml) and the results obtained represent the estimation of biological activity and not its precise value. The confidential limit should be in the range between 64% and 156% of the marked activity. The results obtained are presented below:

Sample	Estimation of biological activity (80-120%)	Conf. limit (64-156%)
FP8 (25°C, 10 weeks)	9095 IU/ml (91%)	69-143%

FP8 (HL, 4 months)	9917 IU/ml (99%)	76-129%

The results show that the estimated biologic activity is in the demanded range and corresponds the demands of Eur. Ph. The confidential limits are also in the demanded range.

The conditions for testing the stability of pharmaceutical compositions which comprise EPO

HL-reference	2 do 8 °C, refrigerator
40	40 °C ± 2°C, 75% relative humidity ± 5%, climatic chamber
25	25 °C ± 2°C, 60% relative humidity ± 5%, climatic chamber

The following examples illustrate the present invention without, however, limiting the same thereto.

Examples

Examples 1-2: The composition of pharmaceutical compositions (FP8) which comprise EPO

Sample	**Active ingredient**	**Inactive ingredient**
FP8 (2000)		
	2000 IU EPO Lek	
		NaH_2P0_4 x2H_2O 1.164 mg
		Na_2HPO_4 x2H_2O 2.225 mg
		NaCl 7.200 mg
		PVP K12 5.000 mg
		NaOH for pH adjustment (pH: 7.0 – 7.1)
		Water to 1 ml

Sample	**Active ingredient**	**Inactive ingredient**

FP8 (10000)		
	10000 IU EPO Lek	
		NaH_2P0_4 x2H_2O 1.164 mg
		Na_2HPO_4 x2H_2O 2.225 mg
		NaCl 7.200 mg
		PVP K12 5.000 mg
		NaOH for pH adjustment pH (pH: 7.0 – 7.1)
		Water to 1 ml

Quality of substances:

Epoetin Lek: quality, demanded by European Pharmacopoeia (Ph. Eur. quality), Povidone K12 (poly[1-(2-oxo-1-pirolidil)ethylene], polyvidone, PVP) Ph Eur. quality, also corresponds to US Pharmacopoeia (USP quality), purchased from BASF, Ludwigshafen, Germany,

NaCl, Na_2HPO_4 x2H_2O, NaH_2P0_4 x2H_2O, NaOH, water for injection: Ph. Eur. quality.

Preparation of pharmaceutical compositions which comprise EPO

Preparation of placebo solution with PVP K12: buffer (Na_2HPO_4 x2H_2O, NaH_2P0_4 x2H_2O), NaCl and stabiliser PVP K12 were dissolved in the water for injection at room temperature by mixing on the magnetic stirrer. pH was then adjusted with 1M NaOH to 7.0–7.1. A clear and colourless solution was obtained.

Preparation of EPO Lek solution: The calculated volume of the EPO Lek solution (calculations were performed regarding the EPO Lek activity) was added to the placebo solution. Just before this step the same volume of placebo solution was withdrawn. The solution was stirred by using a magnetic stirrer at low rotations. A clear colourless solution was obtained.

The solutions of pharmaceutical compositions comprising EPO at both concentrations were then aseptically (air cleanliness level of class 100) filtered through membrane filter with PVDF (Polyvinylidenefluoride) membrane, pore size 0.2 μm, and 0.8 ml of the filtered solutions were filled in 2 ml vials (vials from the

colourless tubular glass hydrolytic type I washed and sterilised, and closed with elastic closures from brombutyl rubber, and sealed with aluminium caps.

Lek Pharmaceuticals d.d.

Patent claims

1. A stable pharmaceutical composition of erythropoietin, which comprises:

a. a therapeutically effective amount of EPO

b. a pharmaceutically acceptable pH buffering system

c. PVP

and optionally further comprises:

d. an isotonifying agent

e. one or more pharmaceutically acceptable excipients

and is free of additives of human and/or animal origin.

2. The pharmaceutical composition according to claim 1 wherein the composition is liquid.
3. The pharmaceutical composition according claim 2 wherein the quantity of EPO is selected in the range of about 500 to about 100,000 IU EPO per dose.
4. The pharmaceutical composition according to claim 3 wherein the quantity of EPO is selected from the group comprising of about 1,000 IU/dose, about 2,000 IU/dose, about 3,000 IU/dose, about 4000 IU/dose, about 10,000 IU/dose, about 20,000 IU/dose, about 25,000 IU/dose or about 40,000 IU/dose.
5. The pharmaceutical composition according to claims 1 to 4 wherein the pH of the solution is in the range from about 6 to about 8.
6. The pharmaceutical composition according to claim 5 wherein the pH of the solution is in the range from about 6.8 to about 7.5.
7. The pharmaceutical composition according to claim 6 wherein the pH of the solution is about 7.0.
8. The pharmaceutical composition according to claims 1-7 wherein the pH buffering system is sodium phosphate buffer.
9. The pharmaceutical composition according to claims 1-8 wherein PVP concentration is in the range from about 0.01% to about 1%.
10. The pharmaceutical composition according to claim 9 wherein PVP is in the range of 0.1% to 1%.
11. The pharmaceutical composition according to claim 10 wherein the concentration of PVP is about 0.5%.

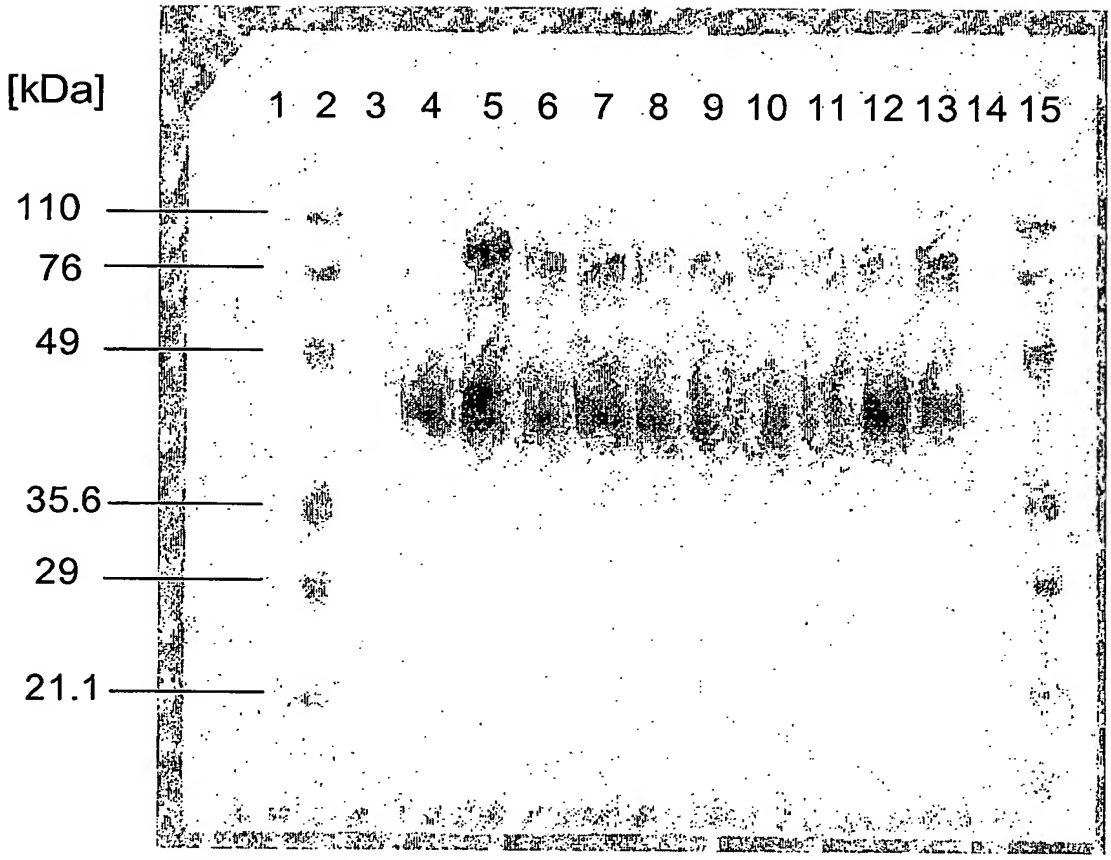
12. The pharmaceutical composition according to claims 1-11 wherein the isotonifying agent is selected from the group of inorganic salts.
13. The pharmaceutical composition of claim 12 wherein the selected isotonifying agent is NaCl.
14. A process for preparing the pharmaceutical composition according to any of claims from 1 to 13.
15. Use of the pharmaceutical composition of any of claims 1 to 14 for the preparation of medicaments useful for the treatment and prevention of diseases indicated for EPO.

Lek Pharmaceuticals d.d.

Abstract

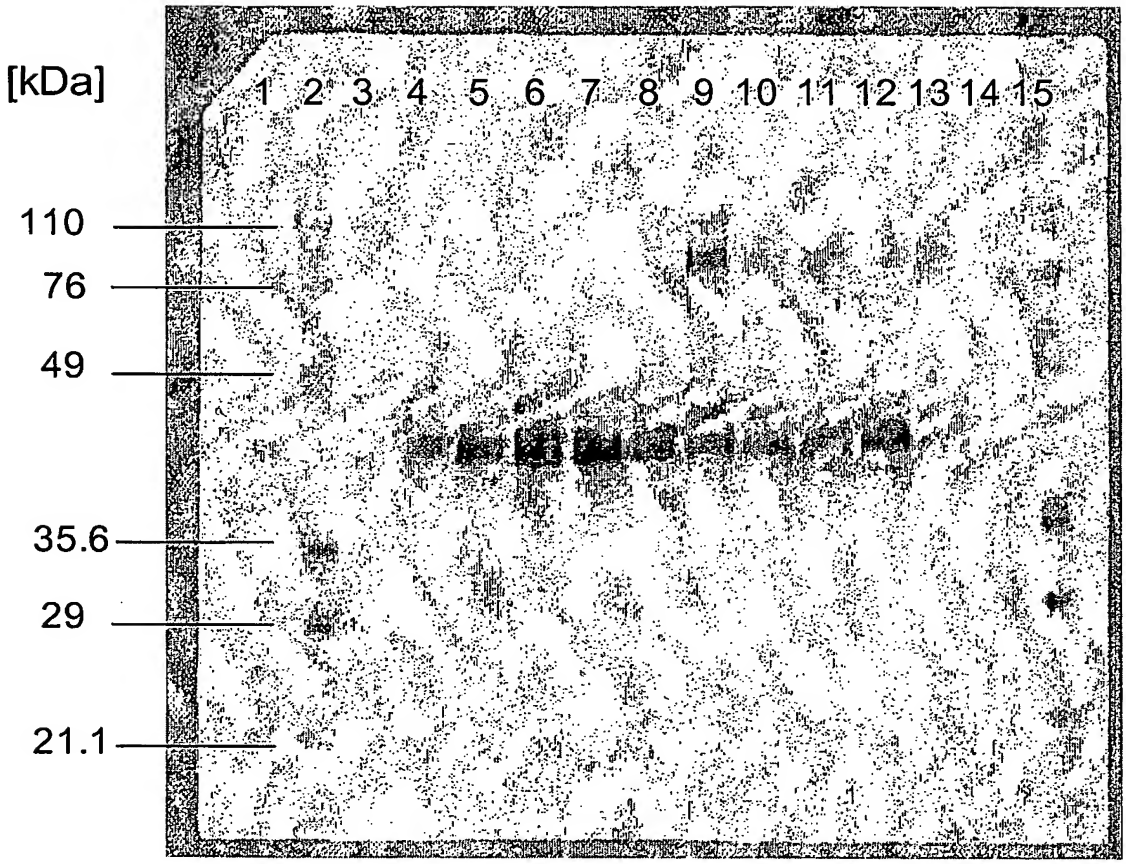
The present invention provides a new stable liquid pharmaceutical composition of erythropoietin that is free of additives of human and/or animal origin.

Figure 1



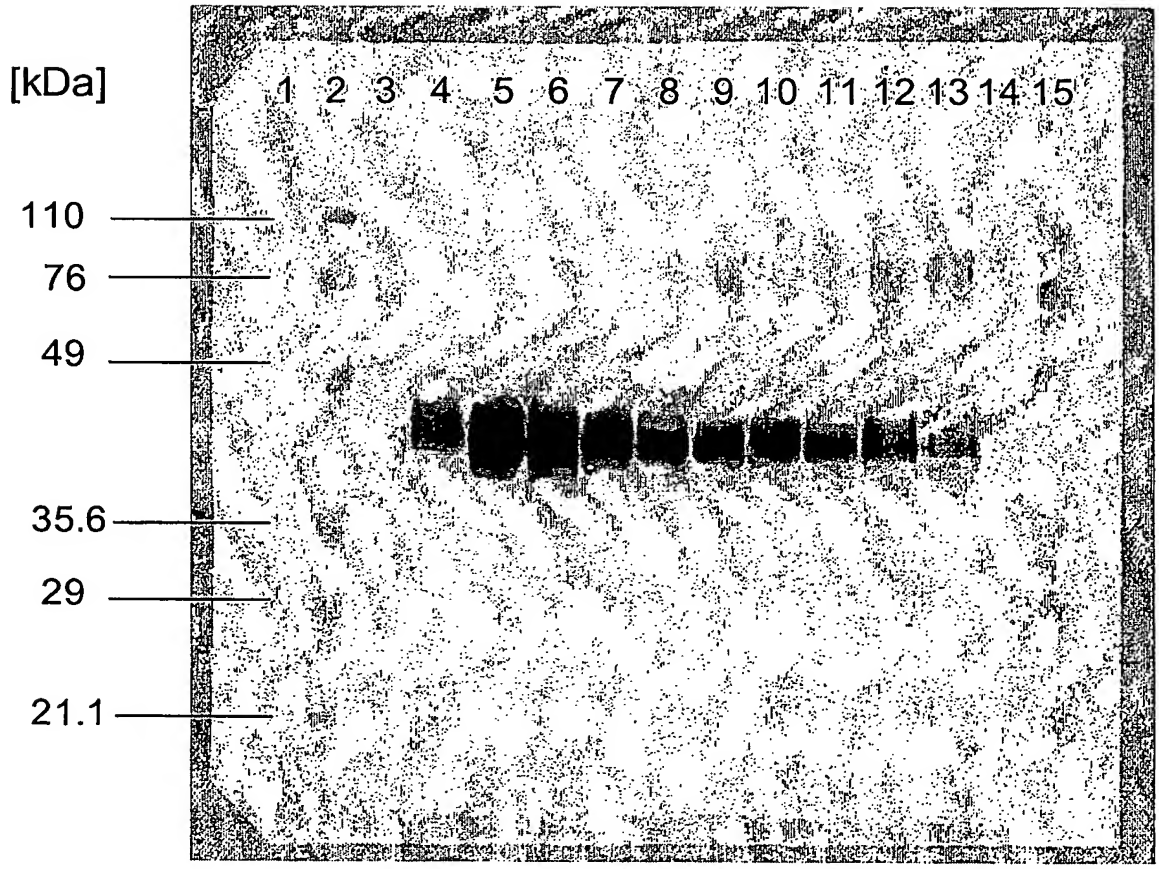
Lek Pharmaceuticals d.d.

Figure 2



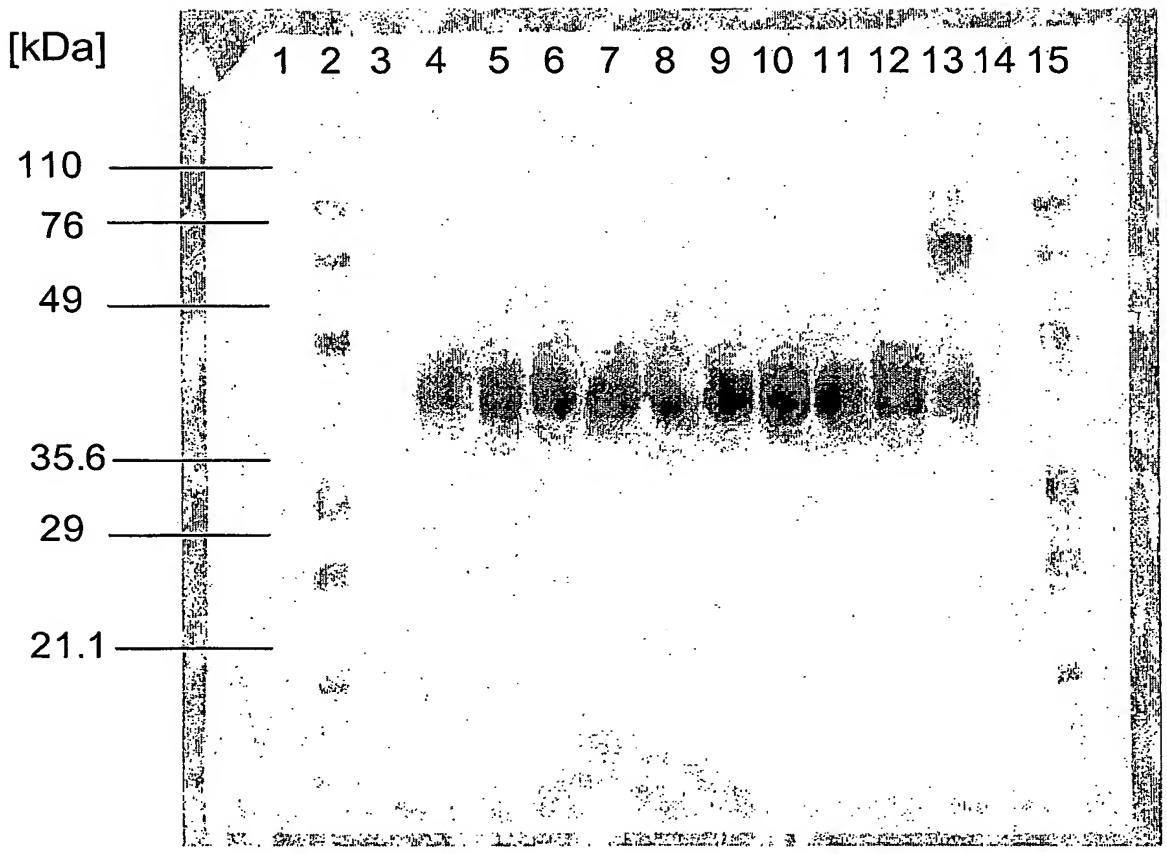
Lek Pharmaceuticals d.d.

Figure 3



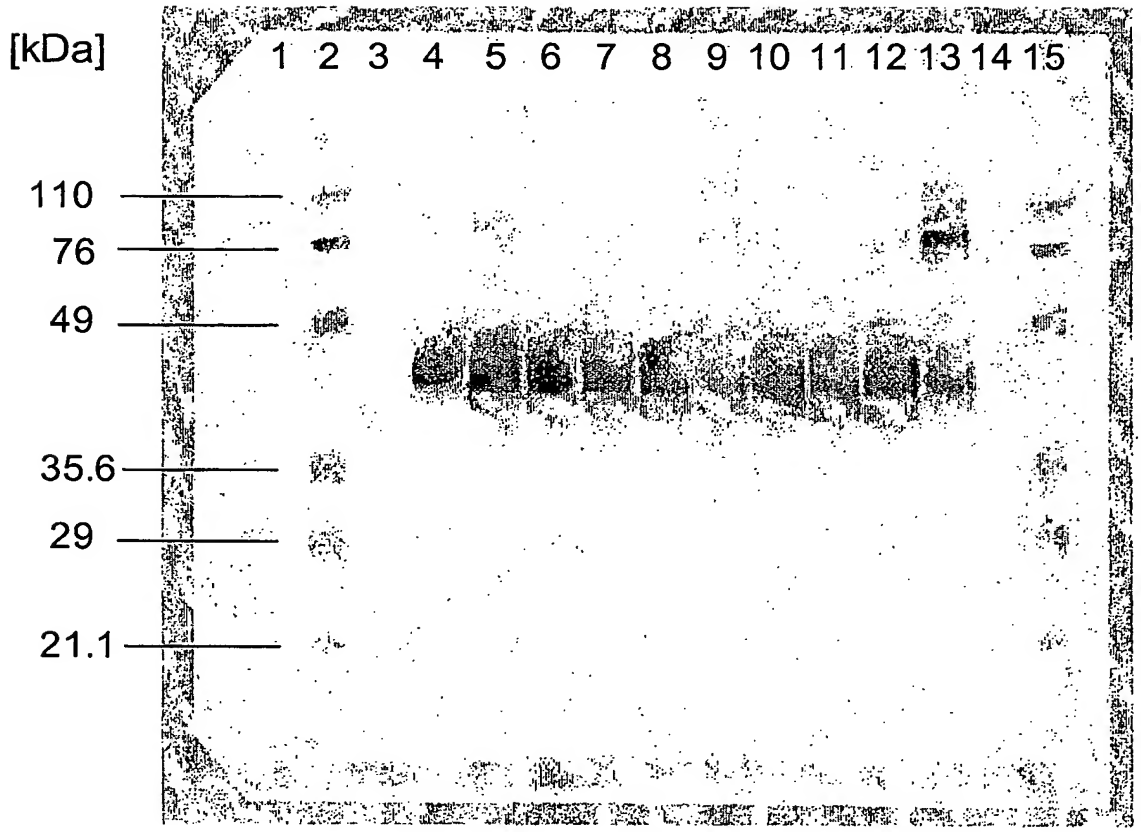
Lek Pharmaceuticals d.d.

Figure 4



Lek Pharmaceuticals d.d.

Figure 5



Lek Pharmaceuticals d.d.

Figure 6

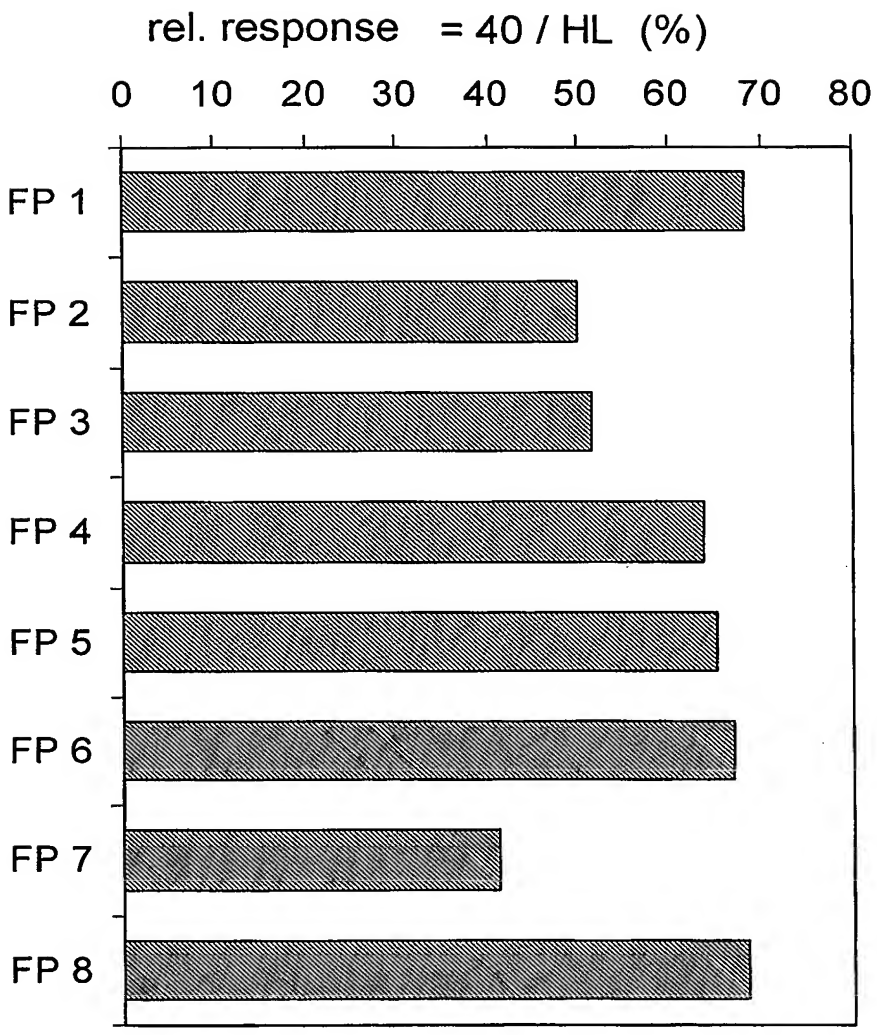


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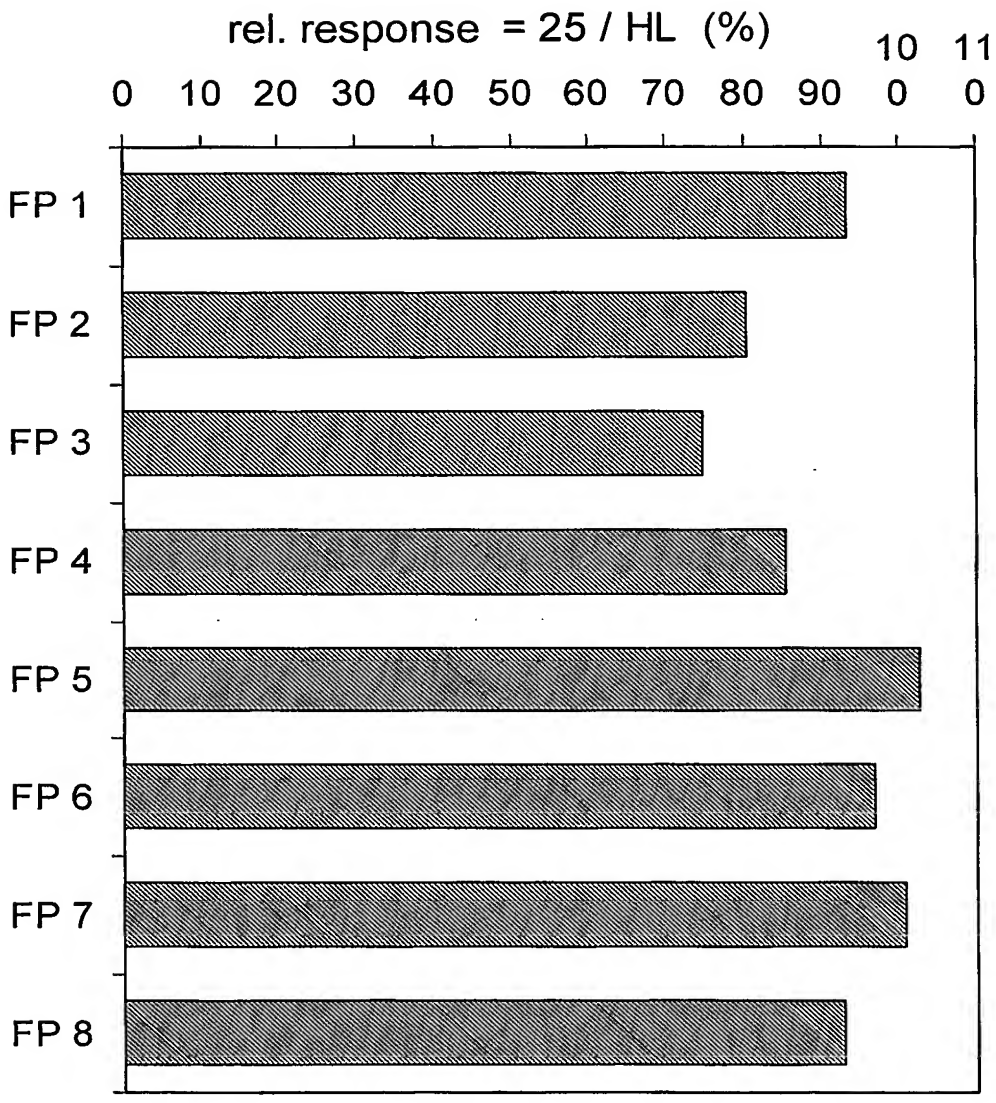
Figure 7



Lek pharmaceuticals d.d.

The undersigned Djurdjica Mandrino, permanent court interpreter for the English language, appointed by Decree No. 756-4/91, issued on 11th of February 1991 by the Ministry of Justice and Administration, Republic of Slovenia, hereby declare that this document entirely corresponds to the original Slovene text.

Ljubljana, 21st October 2002

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